Studies on Microbial Enzymes Acting on Mucin-type Oligosaccharides(Dissertation_全文)

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Contents

Abbreviations

L-BAPA, benzoil-L-arginine *p*-nitroanilide
CD, circular dichroism
ELISA, enzyme-linked immunosorbent assay
endo-α-GalNAc-ase, endo-α-*N*-acetylgalactosaminidase
ESI-MS, electrospray ionization-mass spectrometry
Gal, D-galactose
α-Gal-ase, α-galactosidase
GalNAc, *N*-acetyl-D-galactosamine
α-GalNAc-ase, α-*N*-acetylgalactosaminidase
HPLC, high-performance liquid chromatography
*p*NA, *p*-nitroanilide
*p*NP, *p*-nitrophenyl
PAGE, polyacrylamide gel electrophoresis
PBS, phosphate buffered saline
POD, peroxidase
SDS, sodium dodecyl sulfate
Ser, serine
T antigen, Thomsen-Friedenreich antigen
Thr, threonine
TMBZ, 3,3',5,5'-tetramethylbenzidine
TOF-MS, time of flight-mass spectrometry

General Introduction

Two types of oligosaccharide occur in glycoproteins. One is *N*-linked oligosaccharide attached to the amino-group of asparagine residue of protein, and the other is *O*-linked one attached to the hydroxyl-group of serine (Ser) or threonine (Thr) residue of protein. *O*-Linked oligosaccharides are widely distributed in animals, plants, fungi and yeast. At least, seven different reducing terminal sugars linked to Ser or Thr of various glycoproteins have been described (1). They are *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine, glucose, galactose (Gal), mannose, fucose, and xylose. In eucaryotic microorganisms, especially in yeast *Saccharomyces cerevisiae*, mannose oligomer often bind to Ser or Thr residue. On the other hand, in higher animals, major *O*-linked oligosaccharides of secreted or cell surface glycoproteins are characterized by the core structure of GalNAcα1→Ser/Thr. They are called mucin-type oligosaccharide because they commonly exist in mucins, which are major glycoprotein components of mucus, covering the luminal surfaces of epithelial respiratory, gastrointestinal and reproductive tracts.

Biosynthesis of mucin-type oligosaccharide is initiated by transfer of α-GalNAc from UDP-GalNAc to Ser or Thr of polypeptide catalyzed by polypeptide: α-GalNAc transferases in Golgi apparatus (2). Next, in many cases, β-Gal is transferred to C-3 position of GalNAc residue, resulting in the synthesis of core 1 structure of mucin-type oligosaccharide, Galβ1→3GalNAcα→Ser/Thr. In normal cells, *N*-acetylglucosamine, fucose, sialic acid and sulfuric ester are attached to the core 1 structure of mucin-type oligosaccharide. However, in cancer cells, the core structures, Galβ1→3GalNAcα→ Ser/Thr and GalNAcα→Ser/Thr, are exposed on cell surface glycoproteins. Such unmasked core structures are proposed as tumor associated antigens and are called T (Thomsen-Friedenreich) antigen and Tn antigen, respectively. These antigens are histological markers in carcinoma diagnosis and frequently in prognosis. Moreover, the extent of T and Tn antigens expression often correlates with carcinoma differentiation, and the antigens on carcinoma cell surfaces are suggested to involve in metastasis of cancer (3–4).

In this work, two microbial enzymes acting on the core structures of mucin-type oligosaccharide are studied, which are α-*N*-acetylgalactosaminidase and endo-α-*N*-acetylgalactosaminidase (Fig. 1). These glycosidases are the useful tool for analyses of structure and biological function of mucin-type oligosaccharides attached to glycoproteins. They are also expected to apply to clinical diagnosis *viz.* enzymatic measurement of T and Tn antigen on cancer cells.

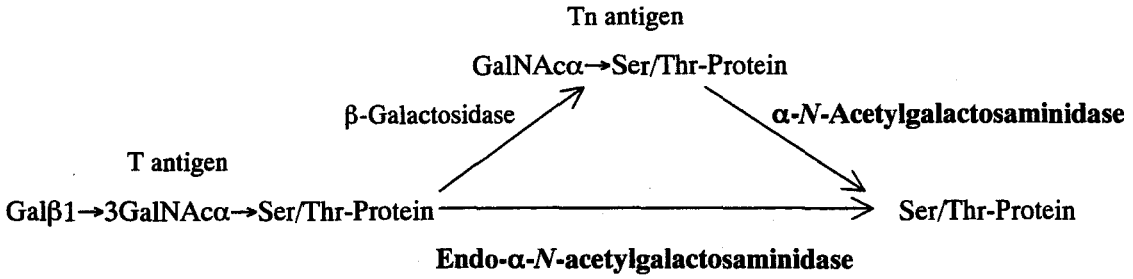

Fig. 1. Glycosidases acting on the core structures of mucin-type oligosaccharides

α-*N*-Acetylgalactosaminidase (α-GalNAc-ase, EC 3.2.1.49) hydrolyze α-linked GalNAc residue of Tn antigen. The enzyme also hydrolyze α-GalNAc residues in the non-reducing terminal of human blood group A substance and mammal (except for primate) Forssman glycolipid. Although the enzyme was obtained from vertebrates, mollucus, fungi and bacteria (5–8), their genes have been reported only from higher vertebrates, such as human, mouse and chicken (9–14). In Chapter I, I am going to describe the gene encoding α-GalNAc-ase from a fungus strain *Acremonium* sp. This is the first report of cloning of its gene from microorganism.

Endo-α-*N*-acetylgalactosaminidase (endo-α-GalNAc-ase, EC 3.2.1.97) catalyzes the hydrolysis of *O*-glycosidic α-linkage between GalNAc and Ser/Thr residue in glycoproteins. The enzyme has hitherto been found from only four kinds of bacteria among all the life, which are *Clostridium perfringens*, *Diplococcus pneumoniae*, *Alcaligenes* sp. and *Streptomyces* sp. (15–20). I have searched for the microorganisms producing the enzyme and isolated a strain belonging to the genus *Bacillus*. In Section 1 of Chapter II, the purification and characterization of the enzyme are described. Further investigation on the properties of the enzyme revealed that it has high transglycosylation activity as well as hydrolysis activity. The transglycosylation activity of endo-type glycosidase is a powerful tool for glycotechnology because intact oligosaccharide can be transferred to a suitable acceptor having hydroxyl group. Using the transglycosylation activity of endo-α-GalNAc-ase from *Bacillus* sp., enzymatic syntheses of various T antigen-containing oligosaccharides and glycolipid mimicry were achieved, which have possibility to prohibit the metastasis of carcinoma. They will be described in Sections 2 and 3 of Chapter II, respectively. In Section 4 of Chapter II, the biological role of mucin-type oligosaccharides of bovine serum fetuin on its trypsin inhibitory activity is elucidated using endo-α-GalNAc-ase.

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Chapter I. Molecular Cloning of the Gene Encoding α-*N*-Acetylgalactosaminidase from *Acremonium* sp. and its Expression in Yeast

INTRODUCTION

α-Linked *N*-acetylgalactosamine (α-GalNAc) residue is widely distributed in various complex carbohydrates such as glycoproteins and glycolipids of higher animals. There are two major α-GalNAc linkages in sugar chains. One exists in the non-reducing end of glycoprotein and glycolipid sugar chains and the other in the reducing end of mucin-type glycoprotein sugar chains. The former is biologically synthesized by human A-enzyme (Gal: α1, 3 GalNAc transferase) (1) or mammal (except for primate) Forssman synthetase (globoside: α1, 3 GalNAc transferase) (2), resulting in intra- and inter-species variation in the oligosaccharide antigens. The latter, GalNAcα1-Ser/Thr, is a common core structure of mucin-type sugar chain in animal glycoproteins and is synthesized by polypeptide: α-GalNAc transferase (3–4).

On the other hand, the hydrolysis of α-GalNAc linkage is catalyzed by lysosomal α-*N*-acetylgalactosaminidase (α-GalNAc-ase, EC 3.2.1.49) in higher animals. The genes of α-GalNAc-ase have been cloned from human (5–6), mouse (7–8) and chicken (9–10). In human, metabolic disorder caused by deficiency of α-GalNAc-ase has been reported as Schindler disease (11) or Kanzaki disease (12), and afflicted individuals excrete much amount of the core structure of mucin-type glycopeptide in urine (13). The human α-GalNAc-ase was first reported as α-galactosidase B (α-Gal-ase B), the isozyme of α-Gal-ase A, because it had a weak though definite activity of α-Gal-ase (14). Later it was found that the gene of α-GalNAc-ase (α-Gal-ase B) had high homology to that of α-Gal-ase A, and both genes were considered to have evolved from a common ancestral gene (6, 15).

As well as in the higher animals, enzyme activities of α-GalNAc-ase have been reported from molluscs (16–19), fungi (20–21) and bacteria (22–25), however, the genes have not been cloned. Kadowaki *et al.* reported the purification and properties of α-GalNAc-ase from the fungus strain *Acremonium* sp. isolated from soil (20). The enzyme was able to convert A red blood cells to O cells (20) and Forssman glycolipid to globoside (26). I confirmed that the enzyme could also hydrolyze GalNAcα1-Ser/Thr linkage which is a core structure of mucin-type sugar chains in glycoproteins (unpublished data). In contrast to vertebrate enzymes, this fungus enzyme has a strict specificity for α-GalNAc residue and hardly hydrolyzes α-Gal linkage, making it useful for structural and functional studies of glycoconjugates. In order to obtain enough pure

enzyme and more information about α-GalNAc-ase, as well as to clarify the evolutionary relationship of this protein, I made an attempt to clone, sequence and express the gene encoding α-GalNAc-ase from *Acremonium* sp.

MATERIALS AND METHODS

Materials. The mRNA purification kit, cDNA synthesis kit, DEAE-Sephadex A-50 and Mono-Q HR 5/5 were purchased from Amersham Pharmacia Biotech. The λgt10 vector and Gigapack III Gold Packing Extract were from Stratagene. DNA modifying enzymes, restriction endonucleases, and the ligation kit were from New England Biolabs, Toyobo Co., Japan, or Takara Shuzo, Co., Japan. [γ-^{32}P]ATP was from ICN Biomedicals Inc. Y-PER yeast protein extraction reagent was from Pierce. Gigapite was from Seikagaku Co., Japan.

Microbial strains and media. *Acremonium* sp. No. 413, isolated from a soil sample (20), was grown on a medium composed of 1 % glucose, 0.5 % peptone, 0.5 % yeast extract and 0.5 % NaCl, pH 6.5 at 28 °C. *Escherichia coli* DH5α and VCS257 were cultivated on LB medium composed of 1 % peptone, 0.5 % yeast extract and 1 % NaCl, pH 7.0 at 37 °C. *Saccharomyces cerevisiae* W303-1A (MATa *ura3-1 leu2-3, 112 trp1 his3-11, 15 ade2-1*) and its derivatives were grown on YPD medium composed of 2 % glucose, 2 % peptone, and 1 % yeast extract, pH 7.0 at 30 °C. For selection of the transformant, a SD plate composed of 2 % glucose and 0.67 % yeast nitrogen base without amino acids (Difco) was used.

Purification of α-GalNAc-ase from Acremonium sp. α-GalNAc-ase was purified from a fungus strain, *Acremonium* sp. No. 413, by the modified method of Kadowaki *et al.* as described previously (20). *Acremonium* sp. was cultivated in a 2-L Sakaguchi flask containing 600 ml of liquid medium for 30 h with shaking. The mycelium were removed by paper filtration and 5 L of supernatant was obtained. The following purification steps were carried out at 4 °C, and unless otherwise indicated, 20 mM potassium phosphate buffer, pH 7.0 (buffer A), was used. The ammonium sulfate precipitate at between 40 % and 70 % saturation was collected by centrifugation and then dissolved in a minimal suitable volume of buffer A and dialyzed against the same buffer. The dialysate (130 ml) was applied to an anion exchange column of DEAE-Sephadex A-50 (3×15 cm) previously equilibrated with buffer A. The column was

washed with the same buffer containing 0.1 M NaCl and the enzyme was eluted with 0.25 M NaCl buffer. The active fractions were collected and dialyzed against 20 mM sodium acetate buffer (pH 6.0). The dialyzed enzyme solution (220 ml) was applied to a hydroxyl apatite column of Gigapite (2×10 cm) previously equilibrated with 20 mM sodium acetate buffer (pH 6.0). The column was washed with the same buffer and the enzyme was eluted with buffer A. The active fractions were collected and applied to an anion exchange column of Mono-Q HR 5/5 previously equilibrated with buffer A. The column was washed with buffer A and eluted with a linear gradient program of 0 to 0.5 M of NaCl in buffer A using the ÄKTA explorer 10S system (Amersham Pharmacia Biotech). The major enzyme activity was eluted with a buffer containing about 0.2 M NaCl and this purified enzyme preparation was used in the study.

Enzyme assay. α-GlcNAc-ase and α-Gal-ase activities were colorimetrically assayed using *p*-nitrophenyl *N*-acetyl-α-D-galactosaminide (*p*NP-α-GalNAc) and *p*-nitrophenyl α-D-galactoside (*p*NP-α-Gal) as the substrate, respectively, as described previously (20). One unit (U) of the enzyme was defined as the amount that hydrolyzes 1 μmol of the substrate per minute.

SDS-PAGE and N-terminal amino acid sequence analysis. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12.5 % acrylamide and 0.1 % SDS with a discontinuous Tris-glycine buffer system (27). Purified enzyme (20 μg) was subjected to SDS-PAGE and electroblotted to Immobilon-P^{SQ} 0.45 μm polyvinylidene fluoride membrane (Millipore). After staining of the membrane with Ponceau S (Sigma), the 57 kDa protein band was cut out and washed with methanol. The membrane strip holding the protein was subjected to automatic Edman degradation for sequencing of the *N*-terminal amino acids using an Applied Biosystems 477A protein sequencer (Parkin Elmer).

Isolation of poly(A) RNA and construction of a cDNA library. *Acremonium* sp. was cultured in the medium described above for 24 h. Fresh mycelium (2 g, wet weight) were frozen with liquid nitrogen and disrupted with a polytron homogenizer. Disrupted cells were suspended in guanidinium thiocyanate solution and total RNA was extracted with water saturated phenol. Poly(A) RNA was obtained from total RNA with an oligo(dT) column (Amersham Pharmacia Biotech). cDNA to the poly(A) RNA was synthesized by cloned Moloney Murine Leukemia Virus reverse transcriptase, and the second strand DNA was synthesized by DNA polymerase. Synthesized cDNA was

blunted by T4 DNA polymerase, and *Eco*RI/*Not*I adapters (Amersham Pharmacia Biotech) were ligated to both ends of the double-stranded cDNA. These cDNAs were purified using a spun column of Sephacryl S-300 (Amersham Pharmacia Biotech), ligated with *Eco*RI/CIAP λgt10 (Stratagene), and in vitro-packaged (Gigapack Gold III, Stratagene) to construct a cDNA library.

Cloning of the gene encoding α-GalNAc-ase from Acremonium sp. A twenty three-residue mixed oligonucleotide probe [5'-CC(GATC)ATGGG(GATC)TT(TC)AA(TC) AA(TC)TGGGC-3'] was synthesized on the basis of the determined *N*-terminal amino acid sequence and used to screen the cDNA library. The oligonucleotide was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The phage plaques including the λgt10 library that appeared on the lawn of *E. coli* VCS257 were transferred to Hybond-N+ filters (Amersham Pharmacia Biotech). After DNA fixation, the filters were hybridized at 55 °C for 36 h with ^{32}P-labeled probe in a buffer containing 3 M TMAC (tetrametylammonium chloride), 5× Denhart's, 0.1 M $NaPO_4$ (pH 6.8), 1 mM EDTA, 100 mg/L denatured salmon DNA and 0.6 % SDS. The filters were washed first with 2× SSC containing 0.1 % SDS at 37 °C for 1 h and then with TMAC wash solution containing 3 M TMAC, 50 mM Tris-HCl (pH 8.0) and 3 % SDS at 37 °C for 30 min. The washed filters were exposed for autoradiography. The cloned phages were amplified in *E. coli* VCS257, and the DNA was extracted and digested with *Eco*RI. The inserted cDNA fragment was subcloned into the *Eco*RI site of plasmid pUC119.

Nucleotide sequence of the α-GalNAc-ase gene. A cDNA fragment containing *nagA* gene was digested by *Msc*I and *Sal*I, and four restriction fragments were subcloned in pUC119 and sequenced in both directions by the method of Sanger *et al.* (28) using a Thermo sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) and Automated DNA sequencer (DSQ-2000L, Shimadzu Co., Japan). The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB037976.

Construction of α-GalNAc-ase gene expression plasmid. Plasmid vector YEGAp was constructed by insertion of GAP (glyceroaldehyde 3-phosphate dehydrogenase) promoter and terminater sequences into the *Hin*dIII site of YEplac112, the *Eco*RI site of which was previously disrupted. The cDNA fragment containing *nagA* was inserted at the *Eco*RI site between the GAP promoter and terminater of YEGAp. *Saccharomyces*

cerevisiae W303-1A was transformed with this expression plasmid by a modified lithium acetate method (29–30) and the transformant selected on the SD plate supplemented with adenine, uracil, histidine, and leucine.

Phylogenetic analysis. The amino acid sequences of α-GalNAc-ase and α-Gal-ase were retrieved from the GenBank and EMBL databases for comparison with the sequence of *Acremonium* α-GalNAc-ase. Sequences were aligned and phylogenetically analyzed using Genetyx Mac Ver. 10 (Software Development Co., Japan). A phylogenetic tree was constructed by the neighbor-joining (NJ) method (31). The tree was evaluated using the bootstrap test (32) based on 1,000 resamplings.

RESULTS

Purification and N-terminal amino acid sequencing of α-GalNAc-ase

α-GalNAc-ase was purified from the culture fluid of *Acremonium* sp. by ammonium sulfate precipitation followed by three steps of column chromatography. The enzyme was purified about 400-fold with 11.6 % recovery (Table I). The final preparation (specific activity = 100 U/mg protein) gave a single protein band of 57 kDa on SDS-PAGE (Fig. 1). To determine the *N*-terminal amino acid sequence, the purified enzyme (20 μg) was subjected to SDS-PAGE and electroblotted to PVDF membrane, then the 57 kDa protein band was analyzed by automatic Edman degradation. The *N*-terminal amino acid sequence was identified as ASQPLLPLPPMGFNNWARF, almost identical with that of mature protein of *aglA* encoding α-galactosidase A from *Aspergillus niger* (33).

Table I. Purification of α-GalNAc-ase from *Acremonium* sp.

	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Culture fluid	3900	936	0.24	1	100
Ammonium sulfate	213	661	3.10	13	70.6
DEAE-Sephadex A-50	28.0	505	18.0	25	54.0
Gigapite	5.95	213	35.8	145	22.8
Mono-Q	1.10	109	99.1	410	11.6

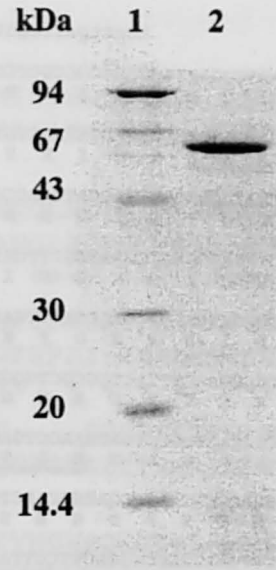

Fig.1 SDS-PAGE of purified α-GalNAc-ase from *Acremonium* sp.
Lane 1, molecular mass standard; lane 2, the purified enzyme.

cDNA cloning and nucleotide sequence of α-GalNAc-ase

A λgt10 cDNA library was constructed from poly(A) RNA of *Acremonium* sp. and 4.3×10^5 plaques were screened with an oligonucleotide mixture probe [5'-CC(GATC) ATGGG(GATC)TT(TC)AA(TC)AA(TC)TGGGC-3'] synthesized on the basis of the *N*-terminal amino acid sequence, PMGFNNWA. Five positive clones were isolated and amplified in *E. coli* VCS257. All the positive clones had an insert fragment of 1.9 kb which was thought to be sufficient to contain the full open reading frame of α-GalNAc-ase. We designated this gene as *nagA*. The cDNA containing *nagA* was sequenced. As shown in Fig. 2, the nucleotide sequence obtained has a length of 1875 bp including a single open reading frame (ORF) with an initiation codon, ATG, and termination codon, TGA, 1641 bases downstream from the first base of the initiation codon. The polyadenylation signal occurred at about 170 bases downstream from the termination codon. The *nagA* gene encodes for 547 amino acid residues in which the sequence from Ala-22 to Phe-30 corresponded completely to the *N*-terminal sequence of the purified native α-GalNAc-ase. Since α-GalNAc-ase is an extracellular enzyme, the *N*-terminal region (Met-1 to Ala-21) could be a signal sequence required for secretion of the enzyme. Two in-frame ATG codons are present in the signal sequence of the α-GalNAc-ase which would result in two different lengths of signal sequence. Three potential *N*-glycosylation sites were found in Asn-43, -83 and -198. The calculated molecular mass of mature protein is 57260 Da, which corresponds to the value obtained from SDS-PAGE of the purified enzyme (57 kDa).

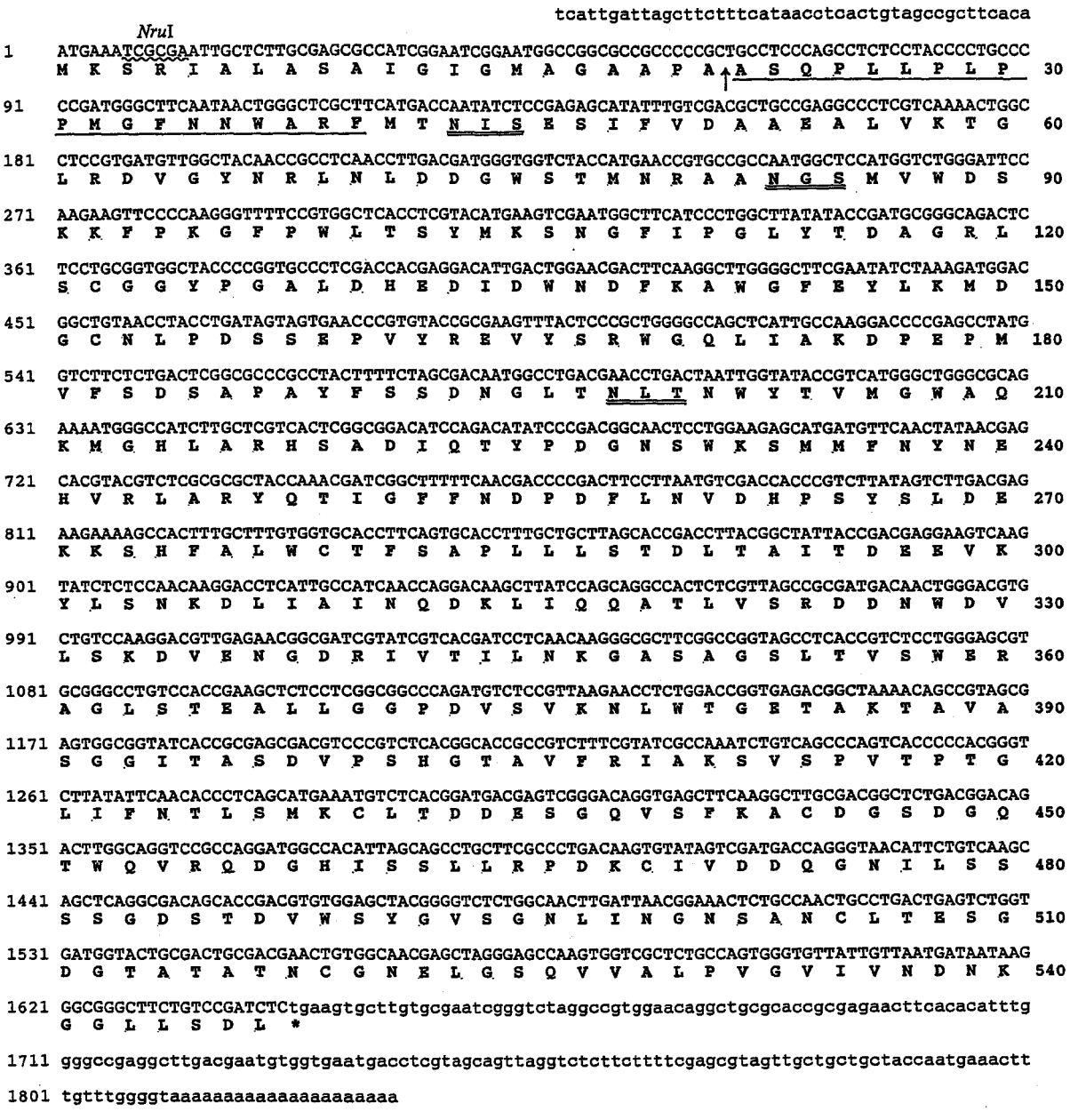

Fig. 2. Nucleotide and deduced amino acid sequences of the *nagA* gene.

The underlined sequence indicates the amino acid sequence determined by peptide sequencing. The arrow indicates the possible cleavage site of the signal peptide. The double underlined sequences indicate the potential *N*-glycosylation site.

```
NagA      MKSRIALASAIGIGMAGAAPA ASQP-LLPLPPMGFNNWARFMTNIS
AglA   MNQGTKRILLAATLAATPWQVYG SIEQPSLLPTPPMGFNNWARFMCDLN
                                  ** *** ************

NagA   ESIFVDAAEALVKTGLRDVGYNRLNLDDGWSTMNRAANGSMVWDSKKFPK
AglA   ETLFTETADTMAANGLRDAGYNRINLDDCWMAYQRSDNGSLQWNTTKFPH
       *  *    *       **** **** **** *    *  ***  *   ***

NagA   GFPWLTSYMKSNGFIPGLYTDAGRLSCGGYPGALDHEDIDWNDFKAWGFE
AglA   GLPWLAKYVKAKGFHFGIYEDSGNMTCGGYPGSYNHEEQDANTFASWGID
       * ***  * *  **  * * * *   ******   **  * * *  **

NagA   YLKMDGCNLPDSS----EPVYREVYSRWGQLIAKDPEPMVFSDSAPAYFS
AglA   YLKLDGCNVYATQGRTLEEEYKQRYGHWHQVLSKMQHPLIFSESAPAYFA
       *** ****          *  *   *  * *   *   *  ** ******

NagA   SDNGLTNLTNWYTVMGWAQKMGHLARHSADIQTYPD-GNSWKSMMFNYNE
AglA   ---GTDNNTDWYTVMDWVPIYGELARHSTDILVYSGAGSAWDSIMNNYNY
          *  * * ***** *    * ***** **  *   *  * * * ***

NagA   HVRLARYQTIGFFNDPDFLNVDHPSYSLDEKKSHFALWCTFSAPLLLSTD
AglA   NTLLARYQRPGYFNDPDFLIPDHPGLTADEKRSHFALWASFSAPLIISAY
          *****  * *******  ***    *** ******  *****  *

NagA   LTAITDEEVKYLSNKDLIAINQDKLIQQATLVSRDDNWDVLSKDVENGDR
AglA   IPALSKDEIAFLTNEALIAVNQDPLAQQATLASRDDTLDILTRSLANGDR
         *    *   * *  *** *** * ***** ****  * *     ****

NagA   IVTILNKGASAGSLTVSWERAGLSTEALLGGPDVSVKNLWTGETAKTAVA
AglA   LLTVLNKGNTTVTRDIPVQWLGLTETDCT----YTAEDLWDGKTQKI---
         * ****              **              ** * * *

NagA   SGGITASDVPSHGTAVFRIAK--SVSPVTPTGLIFNTLSMKCLTDDESGQ
AglA   SDHIKI-ELASHATAVFRLSLPQGCSSVVPTGLVFNTASGNCLTAASNSS
       *  *      ** *****       * * **** *** *  ***

NagA   VSFKACDGSDGQTWQVRQDGHISSLLRPDKCIVDDQGNILSSSSGDSTDV
AglA   VAFQSCNGETSQIWQVTPSGVIRPVSQTTQCLAAD-GNLVKLQACDSTDS
       * *  * *   * ***   * *        *   * **       ****

NagA   ----WSYGVSGNLINGNSANCLTESGDGTATATNCGNELGSQVVALPVGV
AglA   DGQKWTYPVTGNLKNAKTDGCLTE---GSVQMKSCLYERDGQVFGLPSGV
          * * * *** *     ****   *      *  *   **  ** **

NagA   IVNDNKGGLLSDL
AglA   QLA
```

Fig. 3. Sequence alignment of NagA and AglA.

The amino acid sequence of *Acremonium* α-GalNAc-ase (NagA) and *Aspergillus* α-Gal-ase A (AglA) (33) were aligned. Identical amino acids are indicated by asterisks.

Comparison in amino acid sequence between NagA and other related enzymes

To compare the deduced amino acid sequence of the *nagA* gene with the sequences of other genes, the FASTA database (34) was searched. NagA was found to have significant homology (30–45 % identity at the amino acid level) to eucaryotic α-GalNAc-ases and α-Gal-ases, which belong to family 27 of glycoside hydrolase classified by Henrissat (35). Among them, the highest homology was found with α-Gal-ase A (AglA) from *Aspergillus niger* (45 % identity) (33) (see Fig. 3). The hydropathy analyses of these two proteins also gave very similar results in all regions (Fig. 4). A phylogenetic tree of NagA and the major members of family 27 was constructed by the neighbor joining (NJ) method. As shown in Fig. 5, the family 27 enzymes were divided into three clusters; (I) vertebrate α-GalNAc-ases and α-Gal-ases, (II) yeast and fungus α-Gal-ases, and (III) plant and fungus α-Gal-ases. NagA was included in cluster II, and relatively close to the yeast α-Gal-ases, however, it was not so close to the vertebrate α-GalNAc-ases.

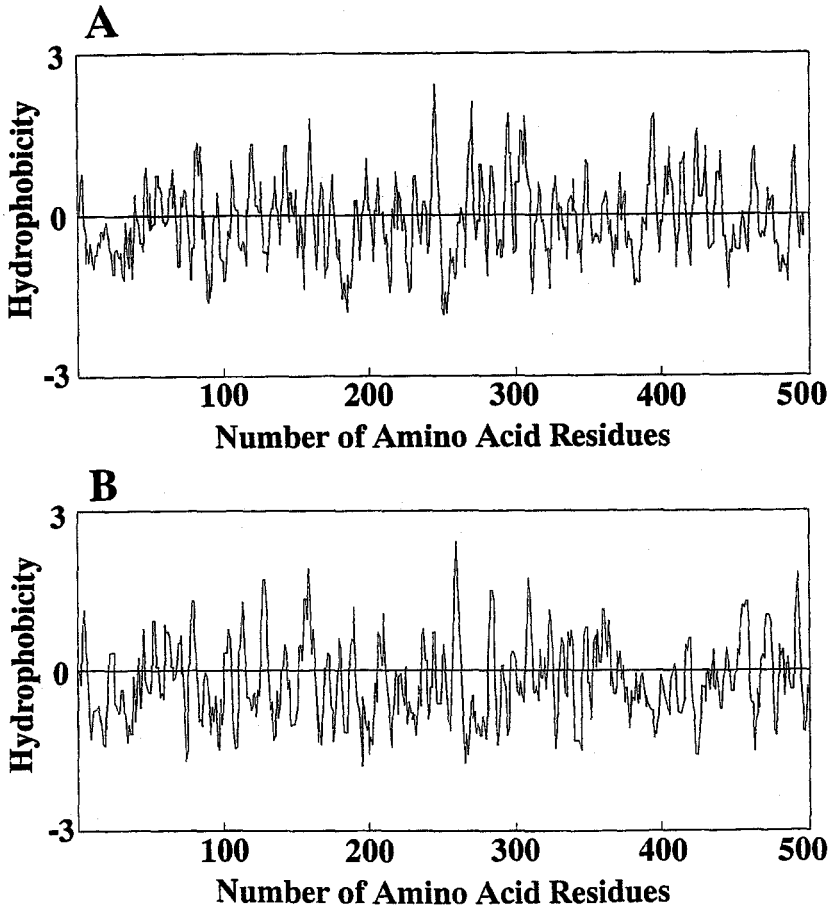

Fig. 4. Hydropathy plots of NagA (A) and AglA (B)

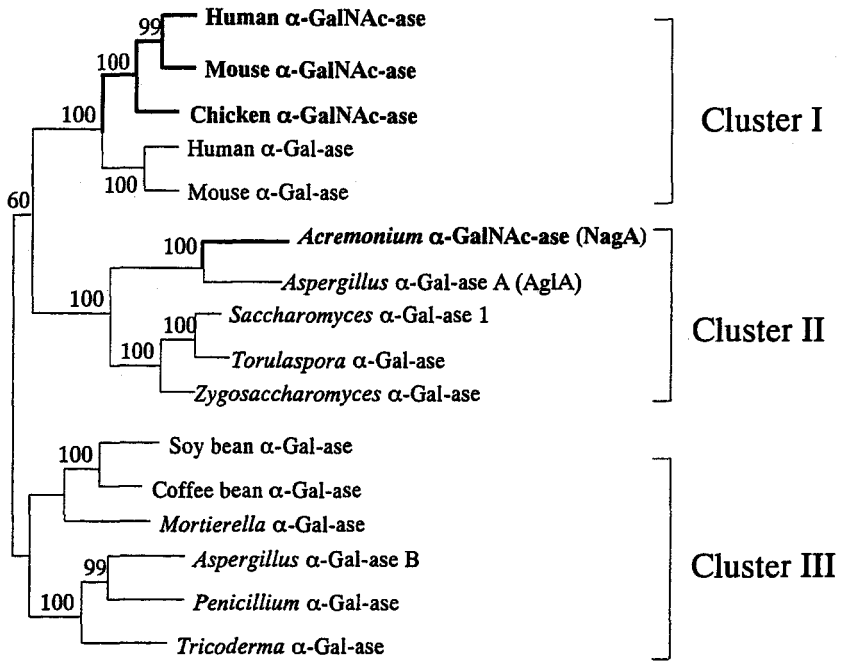

Fig. 5. Phylogenetic relationship between α-GalNAc-ases and α-Gal-ases belonging to the family 27 of glycoside hydrolases (35).

The tree was constructed by the neighbor-joining (NJ) method based on the full length of amino acid sequences. The values at the nodes represent percent of bootstrap confidence levels based on 1,000 resamplings. The accession number of each sequence is as follows: α-GalNAc-ase from human (M38983), mouse (AJ223966), chicken (L18754), *Acremonium* sp. (AB037976, present study); α-Gal-ase from human (M13571), mouse (L46651), soy bean (U12926), coffee bean (L27992), *Mortierella vinacea* (AB018691), *Aspergillus niger* AglA (X63348), AglB (Y18586) , *Penicillium simplicissimum* (AJ009956), *Tricoderma ressei* (S74221), *Saccharomyces cerevisiae* Mel1 (X03102), *Torulaspora delbrueckii* (AB027130), *Zygosaccharomyces cidri* (L24957).

Expression of nagA gene in yeast Saccharomyces cerevisiae

The expression plasmid YEGAp/*nagA* was constructed by insertion of the 1.9-kbp fragment of the coding sequence of *nagA* under the control of the GAP promoter of YEGAp (Fig. 6). *S. cerevisiae* W303-1A was transformed by YEGAp/*nagA* and the transformant was cultured in YPD medium at 30 °C for 70 h. As shown in Table II, α-GalNAc-ase activity was detected in both the culture medium and the cell free extract. Extracellular α-GalNAc-ase activity was 0.5 U/ml, which is 50-fold higher than that of the intracellular fraction, indicating that almost all of the enzyme was secreted into the

culture medium. α-Gal-ase activity was also found in the extracellular fraction, however, it accounted for only 0.55 % of the total α-GalNAc-ase activity of that fraction. Original strain W303-1A and recombinant strains W303-1A (YEGAp) and W303-1A (YEGAp/*nagA*-r, reverse insertion of *nagA*) did not show any activity of α-GalNAc-ase or α-Gal-ase. These results suggested that α-Gal-ase activity in the culture medium of W303-1A (YEGAp/*nagA*) was caused by NagA protein.

Two ATG codons were present upstream of the mature α-GalNAc-ase sequence. Therefore, to identify which codon is needed for expression, plasmid YEGAp/*nagA*-m2 containing 5'-terminal truncated *nagA* was constructed. A cDNA fragment containing *nagA* was digested with *Nru*I to remove the first ATG codon (Fig. 2) and then inserted into YEGAp, for transformation of W303-1A. As shown in Table II, in the transformant, both extracellular and intracellular α-GalNAc-ase activities were decreased to about 1 % of those of yeast cells transformed with YEGAp/*nagA*. This result indicates that the former ATG is an initiation codon.

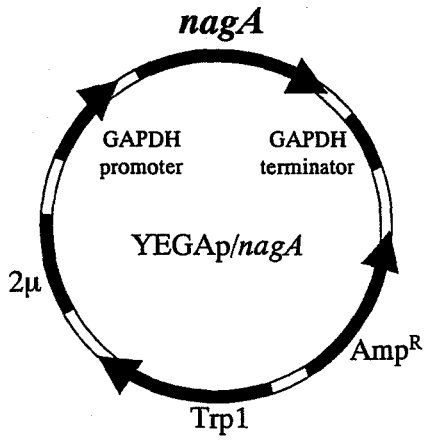

Fig. 6. Plasmid for expression in yeast *S. cerevisiae*.

Table II. Cell growth and enzyme activity of *S. cerevisiae* transformed with various expression plasmids.

Plasmid	Growth (OD610)	α-GalNAc-ase (U/L)		α-Gal-ase (U/L)	
		Extracellular	Intracellular	Extracellular	Intracellular
None	8.7	ND	ND	ND	ND
YEGAp	8.7	ND	ND	ND	ND
YEGAp/*nagA*-r	8.6	ND	ND	ND	ND
YEGAp/*nagA*	8.5	517	9.6	2.8	trace
YEGAp/*nagA*-m2	8.5	25	trace	ND	ND

Note. Yeast strains were cultivated in YPD medium at 30 °C for 72 h. Intracellular enzyme activities were measured by using Y-PER yeast protein extraction reagent (Pierce) and values indicate units per L of culture broth. YEGAp/*nagA*-r, reverse insertion of *nagA*; YEGAp/*nagA*-m2, insertion of first ATG-truncated *nagA*. ND, not detected.

Substrate specificity of recombinant NagA

Substrate specificities of native NagA from *Acremonium* sp. and recombinant NagA from *S. cerevisiae* were compared using natural substrates, human blood group A trisaccharide, GalNAcα1→Ser, and asialo bovine submaxillary mucin. As shown in Table III, both native and recombinant enzymes have almost same specificities. Although blood group A trisaccharide was easily hydrolyzed by NagA, α-GalNAc residue linked to Ser or Thr was hydrolyzed very slowly as compared with commercially available α-GalNAc-ases from animals, such as chicken and squid.

Table III. Substrate specificities of native and recombinant NagA

Substrate	Relative activity (%)			
	Native (*Acremonium*)	Recombinant (*S. cerevisiae*)	Chicken liver	Squid liver
GalNAcα1→*p*NP	100	100	100	100
Blood group A trisaccharide GalNAcα1→3Gal (Fucα1→2)	12.2	13.6	21.6	15.6
GalNAcα1→Ser	0.33	0.38	1.0	0.40
Asialo bovine submaxillary mucin GalNAcα1→Ser(Thr)-protein	0.85	0.99	8.5	4.8

DISCUSSION

In this chapter, I described the molecular cloning of the full length cDNA, *nagA*, encoding α-GalNAc-ase from a fungus strain *Acremonium* sp. and the expression of the gene in yeast *S. cerevisiae*. This is the first report of a microbial α-GalNAc-ase gene. The gene *nagA* codes 547 residues of polypeptide. The amino acid sequence reveals that NagA belongs to family 27 of glycosidases (35). Family 27 consists of α-GalNAc-ases from vertebrate and α-Gal-ases from various eucaryotes such as animals, plants, yeast and fungi. Although NagA has significant homology with α-GalNAc-ases from vertebrates (human, mouse and chicken), the amino acid identity is not so high (32–35 %). Interestingly, NagA has the highest homology with AglA, α-Gal-ase from *A. niger* (45 % identity). The similar results of hydropathy plot analyses also suggest that these two enzyme are closely related. In human and mouse, α-GalNAc-ase and α-Gal-ase genes are considered to be duplicated and evolved from a common ancestral gene, because of their similarity of nucleotide sequence and other gene structures (6, 15). Phylogenetic analysis of family 27 glycosidases revealed that NagA didn't belong to the vertebrate α-GalNAc-ase and α-Gal-ase cluster (cluster I) but to an another cluster (cluster II) including AglA and yeast α-Gal-ases. This suggests that the evolutionary origin of the fungal α-GalNAc-ase is different from that of vertebrate α-GalNAc-ase. I regard this case as an example of parallel evolution. Recently, the presence of α-GalNAc in plant glycoprotein was reported (36). Thus, it is possible that the α-GalNAc-ase of fungal strains inhabiting soil or litter evolved from α-Gal-ase and adapted to metabolite α-GalNAc residues in plants.

Acremonium α-GalNAc-ase in the present study has relatively strict specificity for α-GalNAc residues compared to vertebrate α-GalNAc-ases, which have a broad specificity and considerable α-Gal-ase activity. Therefore, *Acremonium* α-GalNAc-ase is a useful tool for structural and functional studies of glycoconjugate, and the enzyme is now commercially available. I intended to develop a production process for the enzyme using recombinant techniques. To our expectation, NagA was functionally expressed in yeast *Saccharomyces cerevisiae*, and moreover, the gene product was secreted into the extracellular fraction the same as that of *Acremonium* sp. Recombinant enzyme had almost the same substrate specificity for natural substrates as the native enzyme. *N*-terminal amino acid analysis of the native enzyme and the nucleotide sequence of the cDNA revealed that a possible signal peptide could exist. In the putative signal sequence of *nagA*, there are two candidates for an initiation codon, ATG. The results that α-GalNAc-ase activities, both extracellular and intracellular, were markedly

decreased by deletion of the first ATG, suggest that it is the initiation codon with a signal peptide of 21 amino acid residues. Generally, the length of a signal peptide of fungal extracellular protein is 17 or more residues of amino acid (33, 37), which is consistent with our results. When heterologous proteins are expressed in yeast, most are accumulated in the cells. To secrete heterologous protein from *S. cerevisiae*, the signal peptide sequence of yeast invertase or α-mating factor has been used (38-39). It is interesting that the signal peptide of NagA was effective for secretion from yeast cell. The amount of recombinant α-GalNAc-ase secreted from yeast cell was about three times than that of *Acremonium* enzyme, which was calculated to be about 5 mg/L from the specific activity of the purified native enzyme (100 U/mg protein). This value of secreted protein seems to be comparable to the amount obtained when using the signal peptide of invertase or α-mating factor.

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Chapter II. Characterization and Application of Endo-α-*N*-acetylgalactosaminidase from *Bacillus* sp.

Section 1. Purification and Characterization of Endo-α-*N*-acetylgalactosaminidase from *Bacillus* sp.

INTRODUCTION

Endo-α-*N*-acetylgalactosaminidase (endo-α-GalNAc-ase, EC 3.2.1.97) catalyzes the hydrolysis of *O*-glycosidic α-linkage between galactosyl β1, 3 *N*-acetyl-D-galactosamine (Galβ1→3GalNAc) and serine or threonine residue in mucins and mucin-type glycoproteins of various animal sources. This *O*-linked disaccharide is known as the Thomsen-Friedenreich antigen (T antigen) immunodeterminant group used as a specific marker of carcinoma (1–2). The enzyme was first reported from the culture fluid of *Clostridium perfringens* by Huang and Aminoff (3) and then purified from the culture fluids of *Diplococcus pneumoniae* (4–6) and *Alcaligenes* sp. (7–8). They had a strict substrate specificity acting only on the α-linked disaccharide, Galβ1→3GalNAc. However, the aglycone specificities of these enzymes are slightly different (9). A similar enzyme was also found from the culture fluid of *Streptomyces* sp. that could release sugar chains longer than disaccharide from porcine mucin (10–11). As these endo-α-GalNAc-ases liberate *O*-linked oligosaccharide from cell surface glycoproteins without causing damage to cells (12), they are useful for the investigation of the structure and function of *O*-linked oligosaccharides on the cell surface.

It has been reported that several endo-type glycosidases had transglycosylation activity in addition to hydrolysis activity. Transglycosylation activities of endoglycosidases are the powerful tool for glycotechnology because of the whole bioactive oligosaccharide transferring ability.

I searched for microorganisms producing endo-α-GalNAc-ase and isolated a soil bacteria, *Bacillus* sp. A198 strain, producing the enzyme possessing relatively high transglycosylation activity. In this chapter, I describe the purification and characterization of this enzyme.

MATERIALS AND METHODS

Materials. Galβ1→3GalNAcα1→*p*NP, Galβ1→3(GlcNAcβ1→6)GalNAcα1→*p*NP, Galβ1→3GalNAcβ1→*p*NP and Galβ1→3GlcNAcβ1→*p*NP were prepared as described (13). Galβ1→3GalNAcα1→Benzyl, calf fetuin, κ-casein, glycophorin A from blood type MN and submaxillary mucin were purchased from Sigma Co. Neuraminidase was from Nacalai Tesque Co., Japan. Galβ1→3GalNAc was from Toronto Research Chemicals inc., Canada. Actinase E was from Kaken Kagaku Co., Japan. Asialoglycoproteins (asialo-fetuin, asialo-glycophorin and asialo-submaxillary mucin) were prepared from their native glycoproteins by neuraminidase digestion. κ-Casein glycopeptide was prepared from κ-casein by Actinase E digestion. DEAE-Cellulofine A-200 and Gigapite were from Seikagaku Co., Japan. Mini Q PE 4.6/50, Resource S and Superdex 200 HR 10/30 columns were from Amersham Pharmacia Biotech. Toyopearl HW-60C and TSKgel Amide-80 column were from Tosoh Co., Japan. All other chemicals were of the highest grade available from commercial sources.

Microorganism and cultivation. A bacterial strain, A198, which was isolated from a soil sample of Kyoto, Japan, was used throughout this study. This bacterium was Gram positive, rod-shaped and spore-forming, and taxonomically identified as *Bacillus* sp. by referring to Burgey's Manual of Systematic Bacteriology (14). Detailed characteristics will be reported elsewhere.

The bacterium was inoculated into a test tube containing 5 ml of a medium consisting of 0.5 % peptone, 0.5 % yeast extract, 0.5 % meat extract and 0.2 % NaCl (pH 6.5). The culture was carried out for 2 days at 28 °C with shaking, and then the broth was transferred to a 500 ml Sakaguchi flask containing 200 ml of the same medium. The cultivation was continued for 3 days under the conditions described above.

Enzyme assay. The enzyme activity was assayed using Galβ1→3GalNAcα1→*p*NP as the substrate. The reaction mixture composed of 30 μg of substrate (approximately 1.0 mM, final concentration) and a suitable amount of enzyme in 10 mM sodium acetate buffer (pH 5.0) was incubated in a total volume of 60 μl at 37 °C for an appropriate period. The enzyme reaction was stopped by adding 500 μl of sodium borate buffer (pH 10.0), and the released *p*-nitrophenol was measured by absorbance at 400 nm. One unit (U) of the enzyme was defined as the amount of enzyme that hydrolyzes 1 μmol of the

substrate per minute. On the other hand, the disaccharide (Galβ1→3GalNAc) released from the substrate in the reaction mixture was assayed by the method of Reissig *et al.* (15) using pure Galβ1→3GalNAc as the standard. Exo-glycosidase activities including β-galactosidase and α-*N*-acetylgalactosaminidase activities were assayed using various *p*-nitrophenyl glycosides as the substrate. Protease activity was determined using Azocoll as the substrate.

Measurement of protein. The protein concentration was measured using Micro BCA Protein Assay (Pierce, USA) with bovine serum albumin as the standard (16).

HPLC analysis. HPLC analysis was carried out with a Hitachi L-6200 chromatograph with a L-4200 UV-VIS detector. HPLC analysis of oligosaccharides was performed using a normal-phase column (4.6×250 mm, TSKgel Amide-80, Tosoh Co.). Elution was carried out with a solvent of 75 % acetonitrile at a flow rate of 1.0 ml/min at 40 °C, and monitored by absorbance at 214 nm.

ESI-MS analysis. ESI (Electrospray ionization)-mass spectrometry was performed in the positive-ion mode on an Analytica of Branford / JEOL JMS-SX102A mass spectrometer (JEOL, Japan).

Purification of endo-α-GalNAc-ase. All the procedures were carried out at 4 °C. After 3 days-cultivation, 4 liters of culture fluid was obtained after centrifugation at 20,000 *g* for 30 min. The supernatant was brought to 30 % saturation by the addition of solid ammonium sulfate and then stood for 1 day. The resulting precipitate was removed by centrifugation and the supernatant was precipitated by the addition of solid ammonium sulfate to 90 % saturation. The precipitate was collected by centrifugation and then dissolved in a minimal suitable volume of 10 mM potassium phosphate buffer (pH 7.0), followed by dialysis against the same buffer.

The dialysate (200 ml) was applied to anion exchange column of DEAE-Cellulofine A-200 (2.5×25 cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 7.0). The column was eluted with the same buffer and the major enzyme activity was found in the unadsorbed fraction.

The active fractions were collected and brought to 1.5 M by the addition of solid ammonium sulfate, then applied to a hydrophobic interaction column of Toyopearl HW60C (2×10 cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 1.5 M ammonium sulfate. The column was washed with the same

buffer and then the enzyme was eluted with the same buffer containing 0.85 M ammonium sulfate. The active fractions were collected and dialyzed against 10 mM potassium phosphate buffer (pH 7.0).

The dialyzed enzyme solution (20 ml) was subjected to a column (2×5 cm) of Gigapite previously equilibrated with 10 mM potassium phosphate buffer (pH 7.0). The column was washed with 10 mM and 50 mM potassium phosphate buffer (pH 7.0) and the enzyme eluted with 150 mM of the same buffer. The active fractions were concentrated to about 2 ml by ultrafiltration using an Amicon concentrator with a YM-10 membrane (Amicon Co.) and dialyzed against 10 mM Tris-HCl buffer (pH 9.0).

The dialyzed enzyme solution was applied to anion exchange chromatography on a Mini Q PE 4.6/50 column previously equilibrated with 10 mM Tris-HCl buffer (pH 9.0). The column was washed with the same buffer and eluted with a linear gradient program of 0 to 0.5 M of NaCl in the same buffer using the ÄKTA explorer 10S system (Amersham Pharmacia Biotech). The active fractions were collected and dialyzed against 10 mM sodium acetate buffer (pH 5.0).

The enzyme solution was applied to cation exchange chromatography on a Resource S (1 ml) column previously equilibrated with 10 mM sodium acetate buffer (pH 5.0). The column was washed with the same buffer and eluted with a linear gradient program of 0 to 0.5 M of NaCl in the same buffer using the ÄKTA explorer 10S system. The active fraction collected was concentrated to 0.5 ml by Molcut LCC (Millipore).

The enzyme solution was applied to a size exclusion column of Superdex 200 HR 10/30 previously equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 150 mM NaCl. Isocratic elution was carried out using the ÄKTA explorer 10S system. The purified enzyme preparation was obtained and used for the following studies.

SDS-PAGE. SDS-PAGE was performed in 8.0 % polyacrylamide and 0.1 % SDS with a Tris-glycine buffer (pH 8.5) containing 0.1 % SDS. HMW calibration kit proteins for SDS-PAGE (Amersham Pharmacia Biotech) were used as standard for the calibration of the molecular size of the protein. The gel was stained using the kit, 2D-Silver Stain II "DAIICHI" (Daiichi Pure Chemical Co., Japan).

RESULTS

Purification of endo-α-GalNAc-ase from Bacillus sp. A198

Endo-α-GalNAc-ase was purified from the culture fluid of *Bacillus* sp. A198 by ammonium sulfate precipitation, followed by six steps of column chromatography as described in MATERIALS AND METHODS. A purification summary is shown in Table I. The enzyme was purified about 6000-fold with about 5 % recovery. The final preparation of the purified enzyme migrated as a single band on SDS-PAGE (Fig. 1) and the molecular mass of this enzyme was estimated to be 110 kDa by comparison with standard molecular mass proteins. Size exclusion chromatography using the Superdex 200 HR 10/30 column gave almost the same molecular mass. These results indicate that the enzyme has a monomeric structure.

No exo-glycosidase and protease activities were observed in the final preparation.

Table I. Purification of endo-α-GalNAc-ase from *Bacillus* sp.

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Culture fluid	8420	42.1	0.005	1	100
Ammonium sulfate	1230	22.8	0.018	3.7	54.2
DEAE-Cellulofine	233	14.2	0.064	12.8	33.7
Toyopearl HW-60C	15.0	8.64	0.57	115	15.0
Gigapite	1.03	4.80	4.66	932	11.4
Mini Q PE 4.6/50	0.18	2.50	13.9	2780	5.9
Resource S	0.09	2.32	25.8	5160	5.5
Superdex 200 HR 10/30	0.07	2.11	30.1	6020	5.0

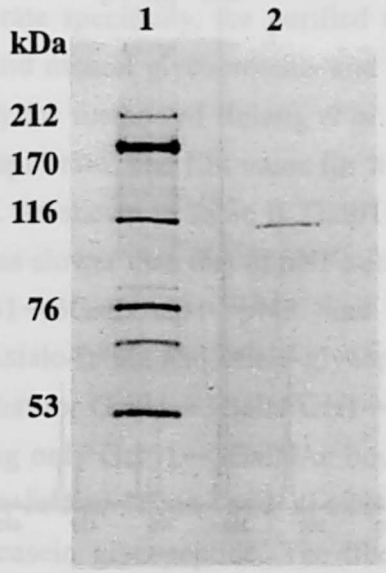

Fig. 1. SDS-PAGE of the purified endo-α-GalNAc-ase from *Bacillus* sp.

The purified enzyme was subjected to SDS-PAGE (8.0% polyacrylamide gel) and stained with silver reagent. Lane 1, molecular mass standards; lane 2, the purified enzyme.

Identification of the disaccharide liberated from asialo-fetuin by endo-α-GalNAc-ase

To identify the reaction product of the enzyme from natural substrate as disaccharide of galactosyl-*N*-acetylgalactosamine, 0.8 mg of asialo-fetuin was incubated with 2 mU of endo-α-GalNAc-ase in 10 mM sodium acetate buffer (pH 5.0) at 37 °C for 1 hr. After incubation, an equal volume of acetone was added to the reaction mixture in order to precipitate fetuin. The mixture was centrifuged and the supernatant was concentrated, then subjected to HPLC using a normal-phase column. Elution was carried out as described in MATERIALS AND METHODS. The peak that corresponded to the reaction product was subjected to ESI-MS analysis in the positive mode. The charged mass ion $[M+H]^+$ was at m/z 383.9 (Fig. 2). The molecular mass of 382.9 calculated from this value coincided with the theoretical value of Galβ1→3GalNAc.

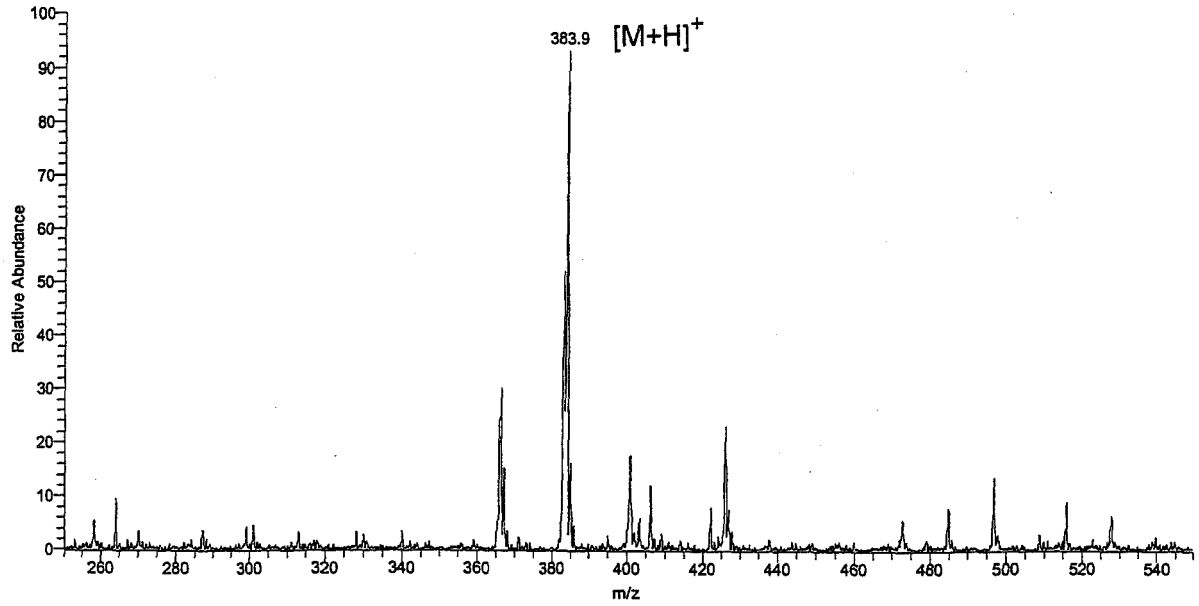

Fig. 2. ESI-MS of disaccharide liberated from asialo-fetuin by the enzyme.

Asialo-fetuin was incubated with endo-α-GalNAc-ase under the condition described in MATERIALS AND METHODS and the liberated sugar chain in the reaction mixture was purified by HPLC before ESI-MS analysis.

Properties of endo-α-GalNAc-ase

The effects of pH on the enzyme activity and stability were examined. Sodium acetate buffer (pH 3.5–6.0), potassium phosphate buffer (pH 6.0–7.5), Tris-HCl buffer (pH 7.5–9.0) and glycine-NaOH buffer (pH 8.5–10.5) were used. Maximal activity of the enzyme was observed at pH 5.0 in 10 mM sodium acetate buffer. The enzyme was stable in the pH range 4.0–10.0 when maintained at 37 °C for 30 min in various buffers (50 mM), and the remaining activity was above 90 % of the original activity in the pH range 5.0–9.0 at 50 °C with 10 min incubation. The enzyme was stable up to about 55 °C in 10 mM sodium acetate buffer (pH 5.0) at 10 min incubation, and for at least 6 months at –20 °C in the same buffer.

Effect of various compounds

The effects of metal ions, chelating reagents and SH-reagents on the enzyme activity were examined. Among various metal ions tested (1 mM of Ca^{2+}, Pb^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+} and Hg^{2+}), Hg^{2+} inactivated the enzyme and Fe^{2+} inhibited it to 70 % of the original activity. EDTA and SH-reagents (CySH, 2-mercaptoetanol and *p*-chloromercuribenzoate) had no effect on enzyme activity.

Substrate specificity

To investigate the substrate specificity, the purified enzyme was incubated with various synthetic substrates and natural glycoproteins and then the amount of released disaccharide was measured by the method of Reissig *et al.* Galβ1→3GalNAcα1→*p*NP was hydrolyzed at the most rapid rate. The Km value for it was calculated to be 50 μM from a Lineweaver-Burk plot. As shown in Table II, Galβ1→3GalNAcα1→Benzyl was also hydrolyzed but its rate was slower than that of *p*NP substrate. Galβ1→3(GlcNAcβ1→6)GalNAcα1→*p*NP, Galβ1→3GalNAcβ1→*p*NP and Galβ1→3GlcNAcβ1→*p*NP were not hydrolyzed at all. Asialo-fetuin and asialo-glycophorin A were hydrolyzed at the rate of 41% and 32% of that for Galβ1→3GalNAcα1→*p*NP, respectively. Asialo-κ-casein glycopeptide containing only Galβ1→3GalNAc bound to threonine residue was hydrolyzed as well as asialo-fetuin. Mono- and di-sialo oligosaccharides were not liberated from fetuin and κ-casein glycopeptide. The liberation of Galβ1→3GalNAc from each substrate was confirmed by TLC using the solvent of 2-propanol: pyridine:H_2O:acetic acid (8:8:4:1, v/v) according to our previous paper (17).

Table II. Substrate specificity of endo-α-GalNAc-ases.

Substrate	Relative activity (%)		
	Bacillus	***Alcaligenes***	***Diplococcus***
Galβ1→3GalNAcα1→*p*NP	100	100	100
GlcNAcβ1↘6 Galβ1→3GalNAcα1→*p*NP	0	0	0
Galβ1→3GalNAcβ1→*p*NP	0	0	0
Galβ1→3GlcNAcβ1→*p*NP	0	0	0
Galβ1→3GalNAcα1→Benzyl	1.6	3.1	< 0.1
Poly (Gal-GalNAc-*p*-aminophenyl-Glu) n	37.0	25.6	7.9
NeuAcα2↘6 Fetuin NeuAcα2→3Galβ1→3GalNAcα1→Ser (Thr) -protein	0	0	0
Asialo fetuin Galβ1→3GalNAcα1→Ser (Thr) -protein	41.2	12.1	23.9
Asialo glycophorine Galβ1→3GalNAcα1→Ser (Thr) -protein	31.8	16.7	33.5
κ-Casein GP NeuAcα2→3Galβ1→3GalNAcα1→Thr-peptide	0	0	0
Asialo κ-casein GP Galβ1→3GalNAcα1→Thr-peptide	34.4	36.2	15.9
Asialo submaxillary mucin GalNAcα1→Ser (Thr) -protein	0	0	0

DISCUSSION

A bacterium strain producing endo-α-GalNAc-ase was isolated from soil and identified as *Bacillus* sp. and the enzyme was purified to homogeneity from the culture fluid of this strain. The substrate specificity of the *Bacillus* enzyme was very similar to that of *Diplococcus* and *Alcaligenes* enzymes that have strict glycone specificity: they can only act on the disaccharide, Galβ1→3GalNAc, bound to aglycone in the α configuration. However, the aglycone specificity of the *Bacillus* enzyme was fairly broad, though that of the other enzymes was not reported sufficiently. *Bacillus* enzyme could hydrolyze not only *p*NP substrate but benzyl substrate, although the hydrolysis rate for the latter was low. The enzyme could also act on the disaccharide of asialo-glycoproteins and glycopeptides such as asialo-fetuin, asialo-glycophorin A and asialo-κ-casein glycopeptide, as well as *p*NP substrate. The enzyme is a useful tool for elucidation of the biological role of mucin-type oligosaccharide on high molecular weight glycoprotein.

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Section 2. Syntheses of Neo-oligosaccharides Using Transglycosylation Activity of Endo-α-*N*-acetylgalactosaminidase

INTRODUCTION

In this decade, it has been reported that several endo-type glycosidases had transglycosylation activity in addition to hydrolysis activity. Transglycosylation activities of endoglycosidases are the powerful tool for glycotechnology because intact oligosaccharide can be transferred. The author and his collaborators have already reported on the transglycosylation activities of microbial endoglycosidases, endo-β-*N*-acetylglucosaminidase (1–3) and endoglycoceramidase (4). They have been used for the synthesis of neo-glycoconjugate, such as bioactive glycopeptides and neo-glycolipids. Concerning endo-α-*N*-acetylgalactosaminidase (α-GalNAc-ase, EC 3.2.1.97), trans-glycosylation and reversed hydrolytic activities have been reported for the *Diplococcus pneumoniae* enzyme (5). Although the syntheses of neo-trisaccharide were achieved by the use of the reversed hydrolytic activity of the *Diplococcus* endo-α-GalNAc-ase incubated with Galβ1→3GalNAc and monosaccharide such as glucose, galactose and fucose, no report has described the synthesis of neo-oligosaccharide using the transglycosylation activity of endo-α-GalNAc-ase which required mono or oligosaccharide and some Galβ1→3GalNAc donor such as asialoglycopeptide.

In previous section, purification and characterization of endo-α-GalNAc-ase from *Bacillus* sp. were described. Further investigation of the properties of the enzyme revealed that it has relatively high transglycosylation activity (Fig. 1). In this chapter, the synthesis of neo-oligosaccharides using the transglycosylation activity of the enzyme is elucidated.

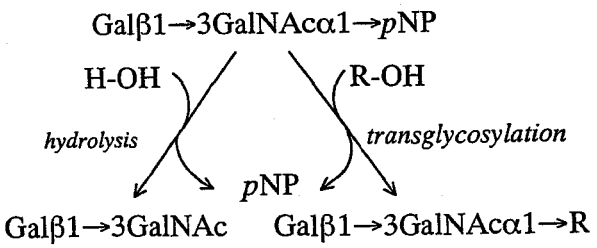

Fig. 1. Transglycosylation activity of endo-α-GalNAc-ase from *Bacillus* sp.

MATERIALS AND METHODS

Materials. Galβ1→3GalNAcα1→*p*NP, was prepared as described (6). Endo-α-GalNAc-ase from *Bacillus* sp. was purified as described in Section 1 of Chapter II.

Enzyme reaction.

The enzyme activity was assayed using Galβ1→3GalNAcα1→*p*NP as the substrate. The reaction mixture composed of 60 μg of substrate (approximately 2.0 mM, final concentration) and 0.2 mU of enzyme in 10 mM sodium acetate buffer (pH 5.0) was incubated in the presence of suitable acceptor (1 M, final conc.) in a total volume of 60 μl at 37 °C for an appropriate period. The enzyme reaction was stopped by boiling and aliquot of reaction mixture was subjected to HPLC analysis

HPLC analysis. HPLC analysis was carried out with a Hitachi L-6200 chromatograph with a L-4200 UV-VIS detector. HPLC analysis of oligosaccharides was performed using a normal-phase column (4.6×250 mm, TSKgel Amide-80, Tosoh Co.). Elution was carried out with a solvent of 75 % acetonitrile at a flow rate of 1.0 ml/min at 40 °C, and monitored by absorbance at 214 nm.

ESI-MS analysis. ESI (Electrospray ionization)-mass spectrometry was performed in the positive-ion mode on an Analytica of Branford / JEOL JMS-SX102A mass spectrometer (JEOL, Japan).

^{1}NMR analyses. One- and two-dimensional ^{1}NMR spectra were measured with a JEOL α-500 spectrometer using NH3XFG as a probe (JEOL, Japan). Chemical shifts are expressed in ppm relative to D_2O as an internal standard.

RESULTS

Transglycosylation reaction of endo-α-GalNAc-ase

To examine the transglycosylation activity of endo-α-GalNAc-ase, a monosaccharide was added to the reaction mixture as an acceptor for Galβ1→3GalNAc released from the substrate. At first, I chose glucose as an acceptor. The reaction mixture was composed of 60 μg of Galβ1→3GalNAcα1→*p*NP (2.0 mM, final conc.) and 0.2 mU of the enzyme in 10 mM sodium acetate buffer (pH 5.0), and incubated in

the absence or presence of 1 M glucose (final conc.) in a total volume of 60 μl at 37 °C. After incubation for 30 min, each reaction mixture was directly analyzed by HPLC using a normal-phase column under the conditions described in MATERIALS AND METHODS. One peak was observed at the retention time of 13.0 min in the reaction mixture without glucose and it seemed to correspond to the hydrolytic product, *p*-nitrophenol. On the other hand, a new peak appeared at the retention time of 25.0 min in addition to the peak of 13.0 min when glucose was added, and the new peak seemed to be the transglycosylation product (Fig. 2). The fraction of the new peak was concentrated and subjected to ESI-MS analysis in the positive mode. The charged mass ion $[M+H]^+$ was at m/z 545.9 (Fig. 3). The molecular mass of 544.9 calculated from this value coincided with the theoretical value of Galβ1→3GalNAc→Glc. These results showed that the disaccharide liberated from Galβ1→3GalNAcα1→*p*NP was transferred to glucose by endo-α-GalNAc-ase.

As the transglycosylation product could be completely hydrolyzed by endo-α-GalNAc-ase (data not shown), the linkage between *N*-acetylgalactosamine and glucose of the product was considered to be an α-bond.

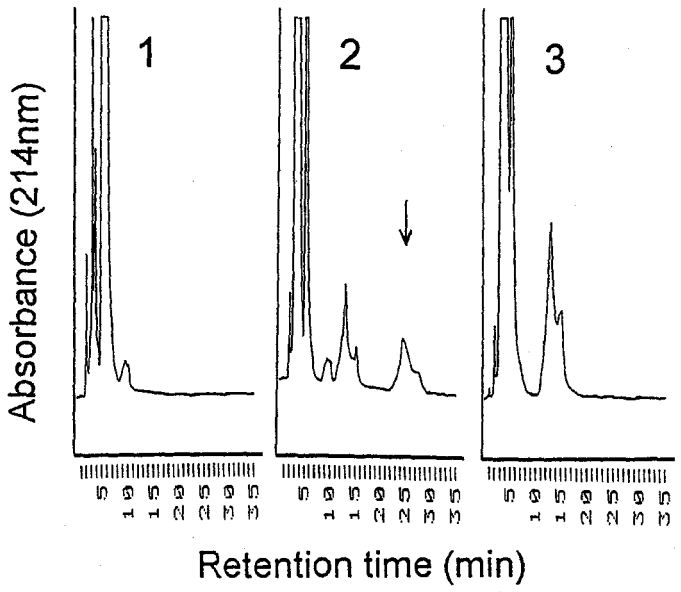

Fig. 2. HPLC profiles of the reaction mixtures with transglycosylation activity of endo-α-GalNAc-ase.

1, Reaction mixture containing *p*NP-disaccharide and glucose without enzyme; 2, reaction mixture containing *p*NP-disaccharide and glucose with enzyme; 3, reaction mixture containing *p*NP-disaccharide and enzyme. The peak around 13 min is the released disaccharide, and the peak indicated by an arrow is a prospective transglycosylation product.

Further structural studies on the transglycosylation product were performed by 500 MHz ^{1}H-NMR analysis. The transglycosylation product (0.3 mg) was subjected to one- and two-dimensional ^{1}H-NMR analyses. As a result, several signals of anomeric proton (H-1 of β-glucose) were observed between 4.5–4.8 ppm and the product was assumed to contain several isomers including Galβ1→3GalNAcα1→2Glc, Galβ1→3GalNAcα1 →1βGlc and Galβ1→3GalNAcα1→1αGlc (data not shown).

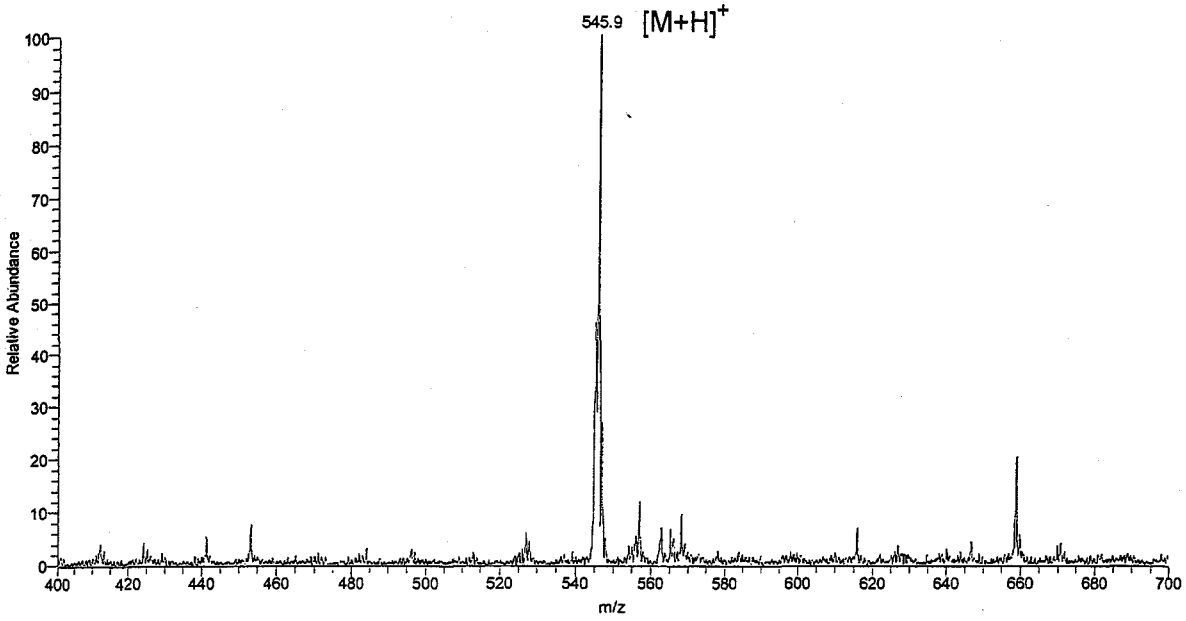

Fig. 3. ESI-MS of the transglycosylation product.

The peak fraction indicated by an arrow in Fig. 2-2 was isolated and analyzed by ESI-MS.

Acceptor specificity of transglycosylation

To examine the acceptor specificity of transglycosylation, various compounds possessing hydroxyl groups were added to the reaction mixture using Galβ1→ 3GalNAcα1→*p*NP as the disaccharide donor. The reaction mixture was of the composition described above except it contained various acceptor compounds instead of glucose, and was incubated in a total volume of 60 μl at 37 °C for 30 min, followed by HPLC the analyses. In each reaction mixture, only one new peak was found and seemed to be the transglycosylation product, judging from its elution position (data not shown). As shown in Table I, monosaccharides such as glucose, galactose and mannose, and sugar alcohols such as sorbitol and mannitol were good acceptors. Disaccharides such

as sucrose and maltose seemed to be also possible acceptors. However, lactose was not because of low solubility.

The effect of pH on the transglycosylation activity was also investigated using glucose as an acceptor. The optimum pH for the activity was 6.0 and the transfer ratio was about 42 %.

Table I. Acceptor specificity of transglycosylation

Acceptor	Transfer ratio (%)*
Glucose	36.4
Galactose	29.3
Mannose	35.5
Mannitol	23.5
Sorbitol	24.8
Maltose	16.5
Sucrose	14.3

**note*. transfer ratio = peak area (PA) of transglycosylation product / (PA of transglycosylation product + PA of hydrolysis product) × 100

DISCUSSION

Bacillus sp. endo-α-GalNAc-ase had transglycosylation activity and could transfer the disaccharide of the substrate to the hydroxyl group of various sugars. When synthetic *p*NP-disaccharide was used as the donor substrate, the enzyme transferred the disaccharide to various monosaccharides, sugar alcohols and disaccharides. Using glucose or galactose as the acceptor, 29–36 % of released disaccharide was transferred to the acceptor during 30 min-incubation. On the other hand, the transglycosylation product was completely hydrolyzed by this enzyme. From this result, the anomeric linkage between the GalNAc moiety of the disaccharide and the acceptor was confirmed to be an α-bond. Further structural studies on the product of transglycosylation to glucose by one- and two-dimensional ^{1}H-NMR analyses showed that the product was assumed to be a mixture of trisaccharide isomers including Galβ1→3GalNAcα1→2Glc, Galβ1→3GalNAcα1→1βGlc and Galβ1→3GalNAcα1→1αGlc.

Although it was reported that the enzyme from *Diplococcus pneumoniae* catalyzed the reversed hydrolysis reaction in addition to the transglycosylation (5), we found no reversed hydrolysis activity of *Bacillus* enzyme.

It is attractive to produce various neo-oligosaccharides and to add the disaccharide to bioactive peptides to produce glycopeptides using the transglycosylation reaction of this enzyme. Further investigation about the production of glycopeptides by the use of this enzyme is underway.

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Section 3. Synthesis of T antigen-containing Glycolipids Mimicry Using Trans-glycosylation Activity of Endo-α-*N*-acetylgalactosaminidase

INTRODUCTION

Many glycosyl hydrolases (glycosidases) have been reported to have transglycosylation and/or reversed hydrolysis activity in addition to hydrolysis activity. Some endo-type glycosidases also have these activities as well as exo-type ones. The author and his collaborators have studied the transglycosylation activity of endo-type glycosidases such as endoglycoceramidase (1), endo-β-*N*-acetylglucosaminidase (2–4), and endo-α-*N*-acetylgalactosaminidase (endo-α-GalNAc-ase) (5), which act on sugar chains of complex carbohydrates. Transglycosylation activities of these endo-glycosidases are a powerful tool for glycotechnology because an intact oligosaccharide can be transferred to a suitable acceptor. In Section 1 of this chapter, the purification and characterization of endo-α-GalNAc-ase (EC 3.2.1.97) from *Bacillus* sp. was described. The enzyme hydrolyzes the *O*-glycosidic α-linkage between Galβ1→3GalNAc and serine or threonine residue in mucin-type glycoproteins (5). In Section 2, I described that the enzyme could transfer the disaccharide, Galβ1→3GalNAc, to mono- or di-saccharides, resulting in the syntheses of neo-oligosaccharides.

The *O*-glycosidic α-linked disaccharide, Galβ1→3GalNAc, is the common core structure of the mucin-type sugar chain and is called the Thomsen-Freidenreich antigen (T antigen) which is well known as carcinoma antigen. T antigen on the normal cell surface is usually masked by sialic acids or neutral oligosaccharides attached at the non-reducing terminus, however, it is exposed on most carcinoma cells of various human tissues such as lung, breast, pancreas, ovarian and bladder (6). Furthermore, T antigen was suggested to mediate adhesion of cancer cells, resulting in tumor metastases or infiltrations (7). Thus, it is speculated that T antigen-containing glycolipid mimicry inhibit the cell adhesion of carcinoma mediated by T antigen, and are promising for anti-metastases of cancer. Besides it, glycolipids are known to be the good immunogen for induction of anti-oligosaccharide antibody. As the tumor-associated oligosaccharide antigens are important targets of cancer vaccine, T antigen-containing glycolipid may be used as a vaccine for T antigen-expressing cancer. For these reasons, I made an attempt to synthesize of T antigen-containing glycolipid mimicry using transglycosylation activity of *Bacillus* endo-α-GalNAc-ase.

MATERIALS AND METHODS

Materials. Galβ1→3GalNAcα1→*p*NP (*p*NP-GalNAc-Gal) was kindly donated from Dr. T. Usui of Shizuoka University. Asialo-fetuin was prepared from bovine serum fetuin (Sigma) by neuraminidase digestion. Various alcohols and detergents were purchased from Nacalai Tesque, Japan. 5-Benzyloxy-1-pentanol and 2-benzyloxy-ethanol were from Aldrich Chem. Mouse anti-T antigen monoclonal antibody (DAKO-HB-T1) from DAKO. POD-conjugated goat anti-mouse IgM antibody was from Sigma. Block Ace was from Dainippon Pharmaceutical, Japan. TMBZ was from Dojindo Lab., Japan.

Enzymes. Endo-α-GalNAc-ase was purified from the culture supernatant of *Bacillus* sp. A198 as described in Section 1 (5). β-Galactosidase and β-*N*-acetyl-hexosaminidase from Jack bean were purchased from Seikagaku Co., Japan. α-*N*-Acetylgalactosaminidase was purified from *Acremonium* sp. as described in Chapter I (8). Neuraminidase from *Arthrobacter ureafaciens* was purchased from Nacalai Tesque, Japan.

Enzyme reaction. The reaction mixture composed of 60 μg of *p*NP-GalNAc-Gal (approximately 2.0 mM, final concentration) and 6 mU of endo-α-GalNAc-ase in 50 mM potassium phosphate buffer (pH 7.0) was incubated with various acceptors (15 %, v/v) in a total volume of 60 μl at 37 °C for an appropriate period. When asialo-fetuin was used as a disaccharide donor, 1.2 mg (20 mg/ml, final concentration) of it was added to the reaction mixture instead of above *p*NP-GalNAc-Gal.

Thin-layer chromatography. The enzyme reaction mixture was analyzed with TLC plate (No. 5547, Merck) using chloroform:methanol:water (65:35:8, v/v) as the developing solvent. Carbohydrate-containing compounds were visualized by spraying with orcinol-H_2SO_4 reagent and heating at 120 °C for 5 min. For quantitative determination, the TLC plate was scanned using HP DeckScan II (Hewlett Packard) and the color intensity of the spots was quantified with imaging software, NIH Image 1.54. The transfer ratio was calculated with the following equation.

$$\text{Transfer ratio (\%)} = \frac{\text{color intensity (CI) of transglycosylation product}}{\text{CI of transglycosylation product + CI of hydrolysis product}} \times 100$$

HPLC analysis. HPLC analysis was performed with a Hitachi L-6200 chromatograph and a L-4200 UV-VIS detector. The transglycosylation products were purified using a reverse-phase column (4.6×150 mm, Cosmocil $5C_{18}$-AR, Nacalai Tesque, Japan). Elution was carried out with a solvent of 10 % acetonitrile in methanol at a flow rate of 0.8 ml/min for 5 min at 40 °C, then with a linear gradient of 10–50 % acetonitrile for 10 min. Elution was monitored by absorbance at 214 nm for hexyl-GalNAc-Gal and at 280 nm for 2-benzyloxyethyl-GalNAc-Gal, respectively.

TOF-MS analysis. TOF (Time of flight)-mass spectrometry was performed in the positive-ion mode on a Voyager Biospectrometry Workstation (PerSeptive Biosystems, USA) using α-cyano-4-hydroxycinnamic acid as the matrix.

ELISA. Fifty μl of asialo-fetuin (500 μg/ml in PBS) was put on a 96-well plate and stored at 4 °C, overnight. After the asialo-fetuin solution in each well was discarded, the plate was washed with PBS, and then 200 μl of Block Ace (diluted 4-times with PBS) was added and incubated for 2 h at room temperature. Blocking solutions were discarded and the plate was washed with PBS containing 0.1 % Tween 20. Then, 50 μl of anti-T antigen monoclonal antibody solution (DAKO-HB-T1, 15 μg/ml in PBS), and various amounts of T antigen-containing glycolipid mimicry were added and incubated at room temperature for 3 h. POD-conjugated goat anti-mouse IgM (diluted 10,000-fold with PBS) was used as the secondary antibody, and TMBZ and H_2O_2 were used as substrate for POD. The enzyme reaction was carried out in 100 mM sodium acetate buffer (pH 5.5) containing 0.1 % Tween 20 at 30 °C for 30 min, and then absorbance was measured at 450 nm.

RESULTS

Transglycosylation of disaccharide from pNP-substrate to 1-hexanol

To examine the transglycosylation activity of *Bacillus* endo-α-GalNAc-ase to 1-alkanol, 2 mM *p*NP-GalNAc-Gal, and 100 mU/ml of the enzyme were incubated with 15 % (v/v) 1-hexanol as the acceptor in the absence or presence of 0.2 % (w/v) sodium cholate. After incubation at 37 °C for 3 h, the reaction mixtures were analyzed by TLC using chloroform:methanol:water (65:35:8, v/v) as the developing solvent. The transglycosylation product could be hardly detected by TLC when detergent was absent from the reaction mixture. However, a new product was clearly found (Rf value = 0.43),

when sodium cholate was added in the reaction mixture (Fig. 1). To isolate the product, the part of TLC plate corresponding to the mobility of product was taken off and extracted with chloroform:methanol (2:1). Then, the extract was applied to the reverse-phase column on HPLC. Sugar-containing compound gave a single peak, and the fractions of it were collected and lyophilized. The purified product was subjected to TOF-MS analysis in the positive mode. The charged mass ions $[M+Na]^+$ and $[M+K]^+$ were found at m/z 490.4 and 506.4, respectively (Fig. 2). The molecular mass of 467.4 calculated from these values coincided with the theoretical value of Galβ1→3GalNAcα1→hexyl (hexyl-GalNAc-Gal). These results showed that the disaccharide liberated from *p*NP-GalNAc-Gal was transferred to 1-hexanol by *Bacillus* endo-α-GalNAc-ase.

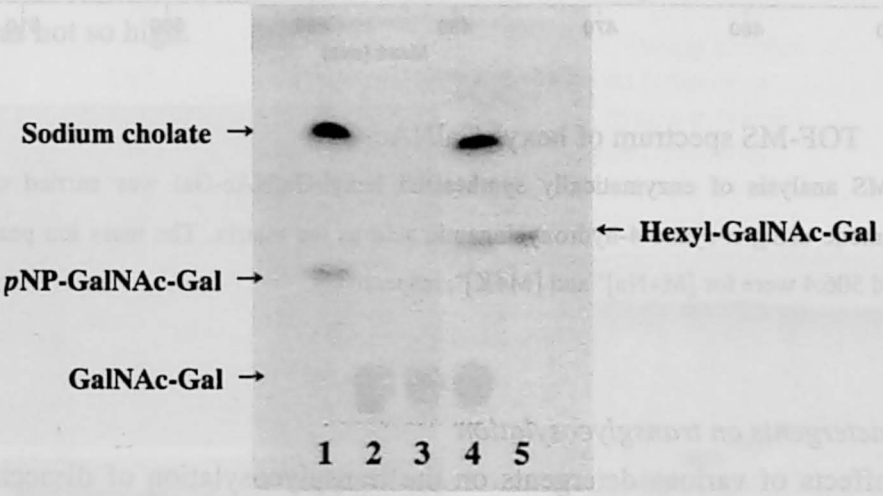

Fig. 1. Transglycosylation of disaccharide from *p*NP-GalNAc-Gal to 1-hexanol by endo-α-GalNAc-ase.

The reaction mixtures were analyzed by TLC using chloroform:methanol:water (65:35:8, v/v). Lane 1, *p*NP-GalNAc-Gal + sodium cholate; lane 2, *p*NP-GalNAc-Gal + enzyme; lane 3, *p*NP-GalNAc-Gal + 1-hexanol + enzyme; lane 4, *p*NP-GalNAc-Gal + 1-hexanol + sodium cholate + enzyme; lane 5, purified hexyl-GalNAc-Gal.

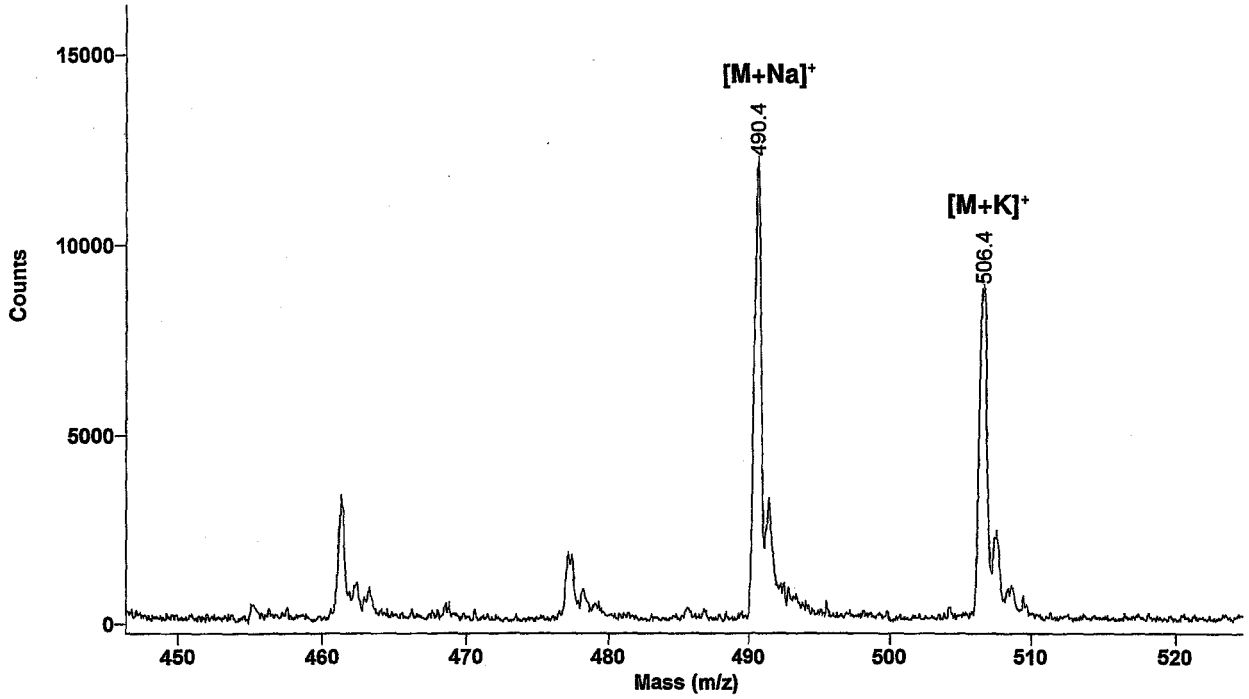

Fig. 2. TOF-MS spectrum of hexyl-GalNAc-Gal.

TOF-MS analysis of enzymatically synthesized hexyl-GalNAc-Gal was carried out in the positive mode using α-cyano-4-hydroxycinnamic acid as the matrix. The mass ion peaks of m/z 490.4 and 506.4 were for $[M+Na]^+$ and $[M+K]^+$, respectively.

Effects of detergents on transglycosylation

The effects of various detergents on the transglycosylation of disaccharide to 1-hexanol by endo-α-GalNAc-ase were investigated. The detergents tested were Triton X-100, Tween 20, Tween 80, sodium cholate, sodium deoxycholate, and sodium dodecyl sulfate (SDS). Each was added to the reaction mixture at a concentration of 0.2 % (w/v). Among the detergents tested, sodium cholate gave the highest yield of transglycosylation product (60 % in transfer ratio). Sodium deoxycholate and nonionic detergents (Triton X-100, Tween 20 and Tween 80) were slighty effective (20–30 %). SDS strongly inhibited the enzyme activity, thus, neither a hydrolysis or transglycosylation product was detected.

Acceptor specificity for transglycosylation

Acceptor specificity was examined using various 1-alkanols as the acceptor with or without sodium cholate. As shown in Fig. 3-A and B, the corresponding transglycosylation products from the various 1-alkanols were found on TLC. The color

intensity of each spot was quantified with an image scanner and the results are shown in Table I. When sodium cholate was not added to the reaction mixture, about 70 % of the disaccharide from *p*NP-GalNAc-Gal was transferred to methanol, ethanol, 1-propanol and 1-butanol. But when the carbon number of 1-alkanol was more than five, the yield of transglycosylation product decreased. When sodium cholate was added, the degree of transglycosylation of disaccharide to short chain alkanols was the same as that without sodium cholate, but the transglycosylation to long chain alkanols (C_5–C_8) was more effective than that without sodium cholate.

In addition to 1-alkanols, other alcohols were also tested for the transglycosylation of disaccharide. The results are shown in Table I. Allyl alcohol, etylen glycol monoethyl ether and dietylen glycol monoethyl ether, which are soluble in water, were good acceptors and their disaccharide transfer ratios were more than 70 %. 2-Benzyloxyethanol and 5-benzyloxypentanol were also accepted but the transfer ratio of the latter was not so high.

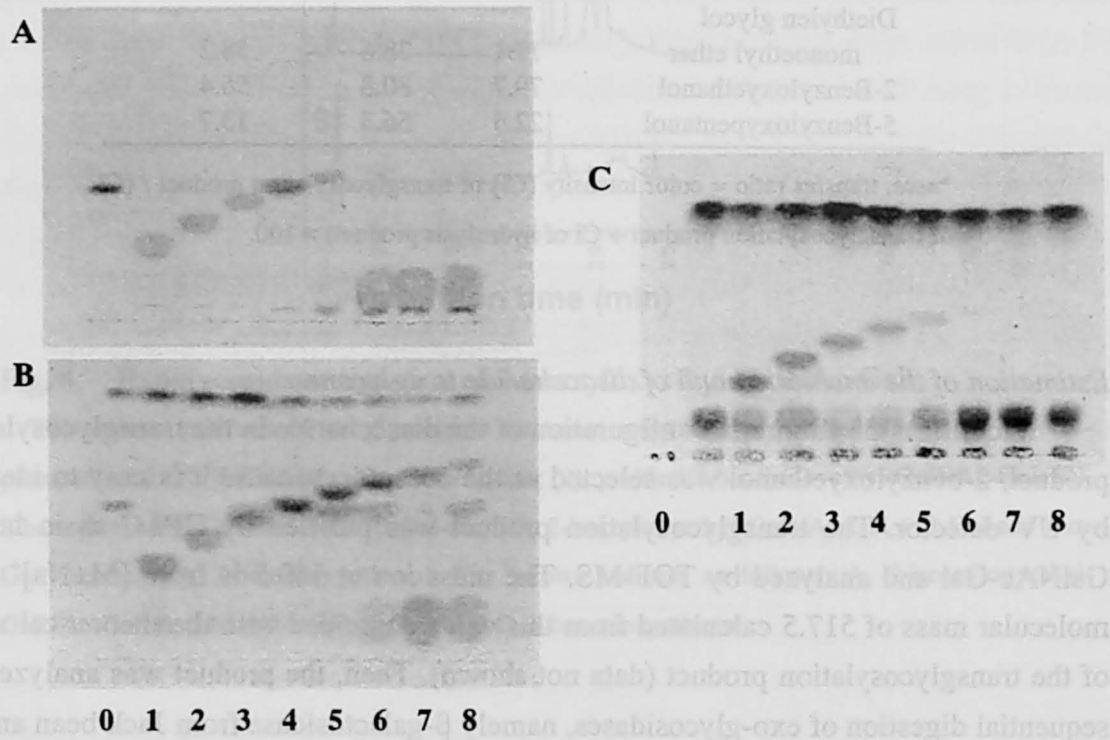

Fig. 3. Transglycosylation of disaccharide to various 1-alkanols by endo-α-GalNAc-ase.

A, *p*NP-GalNAc-Gal + 1-alkanol + enzyme; B, *p*NP-GalNAc-Gal + 1-alkanol + sodium cholate + enzyme; C, asialo-fetuin + 1-alkanol + sodium cholate + enzyme. Lane 0, donor only; lane 1, add methanol as acceptor; lane 2, ethanol; lane 3, 1-propanpl; lane 4, 1-butanol; lane 5, 1-pentanol; lane 6, 1-hexanol; 7, 1-heptanol; 8, 1-octanol.

Table I. Acceptor specificity for transglycosylation of disaccharide.

Acceptor	Donor cholate	Transfer ratio (%)* *p*NP-GalNAc-Gal −	*p*NP-GalNAc-Gal +	asialo-fetuin +
Methanol (C1)		71.3	70.8	56.0
Ethanol (C2)		65.8	65.0	50.1
1-Propanol (C3)		69.8	76.8	63.3
1-Butanol (C4)		76.8	85.6	48.4
1-Pentanol (C5)		28.2	73.3	18.9
1-Hexanol (C6)		7.5	58.8	3.8
1-Heptanol (C7)		3.1	22.7	trace
1-Octanol (C8)		trace	14.9	trace
Allyl alcohol		85.1	86.8	60.2
Ethylen glycol monoethyl ether		79.1	79.4	59.5
Diethylen glycol monoethyl ether		77.1	78.6	58.2
2-Benzyloxyethanol		79.7	80.8	56.4
5-Benzyloxypentanol		22.5	56.3	13.7

***note.* transfer ratio = color intensity (CI) of transglycosylation product / (CI of transglycosylation product + CI of hydrolysis product) × 100.

Estimation of the anomeric bond of disaccharide to aglycone

To confirm the anomeric configuration of the disaccharide in the transglycosylation product, 2-benzyloxyethanol was selected as the acceptor, because it is easy to identify by UV detector. The transglycosylation product was purified by HPLC as in hexyl-GalNAc-Gal and analyzed by TOF-MS. The mass ion at 540.5 is from $[M+Na]^+$. The molecular mass of 517.5 calculated from this value coincided with the theoretical value of the transglycosylation product (data not shown). Then, the product was analyzed by sequential digestion of exo-glycosidases, namely β-galactosidase from Jack bean and α-*N*-acetylgalactosaminidase from *Acremonium* sp. Each reaction mixture was subjected to HPLC with a reversed phase column. As shown in Fig. 4, the transglycosylation product was completely hydrolyzed on addition of these exo-glycosidases. On the other hand, the product hydrolyzed with β-galactosidase was not degraded by β-*N*-acetylhexosaminidase from Jack bean (data not shown). These results indicate that the anomeric bond of the transglycosylation product is an α-configuration.

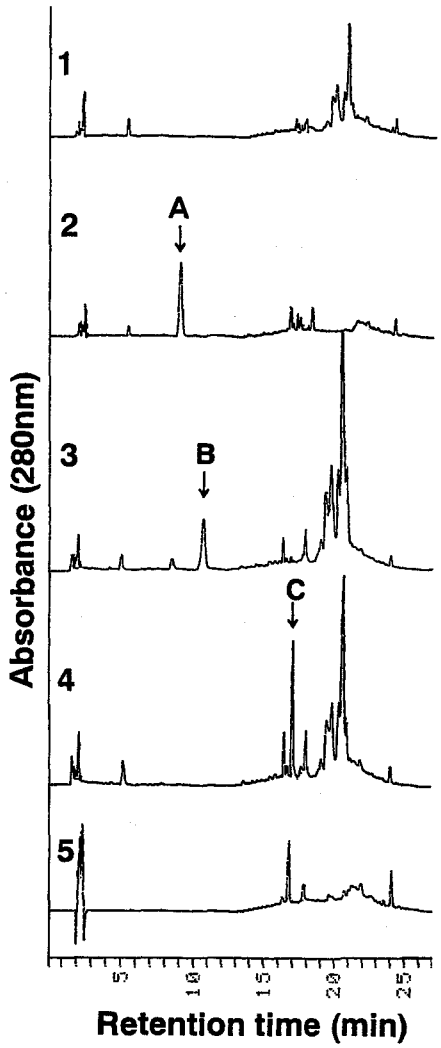

Fig. 4. Exoglycosidase digestion of 2-benzyloxyethyl-GalNAc-Gal.

The reaction mixtures of exoglycosidase treatment were analyzed with HPLC on a reverse-phase column. (1), β-Gal-ase and α-GalNAc-ase; (2), purified 2-benzyloxyethyl-GalNAc-Gal; (3), 2-benzyloxyethyl-GalNAc-Gal + β-Gal-ase; (4), 2-benzyloxyethyl-GalNAc-Gal + β-Gal-ase + α-GalNAc-ase; (5), 2-benzyloxyethanol. The arrows indicate as follows: A, 2-benzyloxyethyl-GalNAc-Gal; B, 2-benzyloxyethyl-GalNAc; C, 2-benzyloxyethanol.

Transglycosylation of disaccharide from native glycoprotein

To examine the transferring activity of endo-α-GalNAc-ase using native glycoprotein as a donor of disaccharide, neuraminidase-treated bovine fetuin (asialo-fetuin) was used. Twenty mg/ml asialo-fetuin and 100 mU/ml endo-α-GalNAc-ase were incubated with various 1-alkanols (15 %, v/v) as the acceptor in the presence of 0.2 % sodium cholate. After incubation at 37 °C for 6 h, the reaction mixtures were evaporated

and extracted with chloroform:methanol (2:1), and then assayed with TLC. As shown in Fig. 3-C, the disaccharide was transferred very well to methanol, ethanol, 1-propanol and 1-butanol (transfer ratio = 50–65 %), and also to 1-pentanol (20 %). However, little transfer occured with 1-hexanol, and none with 1-heptanol or 1-octanol. In spite of the presence of sodium cholate, the transfer ratios from asialo-fetuin to 1-alkanols were lower than those from *p*NP-substrate to 1-aklanols even without sodium cholate.

Inhibition of binding of anti-T antigen antibody to asialo-fetuin by glycolipid mimicry

The inhibitory activity of T antigen-containing glycolipids mimicry for binding of anti-T antigen monoclonal antibody to asialo-fetuin was investigated. To evaluate the inhibitory activity, ELISA was carried out. Mouse anti-T (DAKO-HB-T1) and various amounts of Galβ1→3GalNAcα1→hexyl (hexyl-GalNAc-Gal), Galβ1→3 GalNAc (free T disaccharide), or GlcNAcβ1→hexyl (hexyl-β-GlcNAc) were added to a 96-well plate previously coated with asialo-fetuin. As shown in Fig. 5, the control compound, hexyl-β-GlcNAc, did not inhibit the binding at all, while free T disaccharide inhibited it to a certain degree. However, enzymatically synthesized hexyl-GalNAc-Gal inhibited the binding 100-times stronger than free T disaccharide.

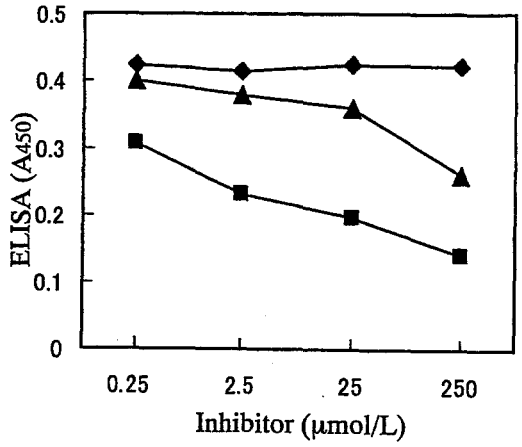

Fig. 5. Inhibition of binding of anti-T antigen antibody to asialo-fetuin by hexyl-GalNAc-Gal.

Anti-T antigen monoclonal antibody and various concentrations of inhibitor were incubated in the well previously coated with asialo-fetuin. Each point represents the mean of three determinations. Symbols: ■, Galβ1→3GalNAcα1→hexyl; ▲, Galβ1→3GalNAc; ◆, GlcNAcβ1→hexyl.

DISCUSSION

In this section, I described the syntheses of T antigen-containing glycolipid mimicry using the transglycosylation activity of endo-α-GalNAc-ase from *Bacillus* sp. This is the first report of enzymatic synthesis of neo-glycolipid having mucin-type oligosaccharide. In addition to the *Bacillus* enzyme used in this study, there are two commercially available endo-α-GalNAc-ases from microbes, are *Diplococcus pneumoniae* (9–10) and *Alcaligenes* sp. (11). These three enzymes have the same specificity for glycone, which is α-linked Galβ1→3GalNAc, but differ slightly in their specificities for aglycone, *viz.*, *Bacillus* enzyme has broader aglycone specificity than the others (5). It was reported that *Diplococcus* enzyme catalyzed the reversed hydrolysis reaction as well as the transglycosylation. Although the syntheses of neo-oligosaccharides and amino-disaccharides were achieved using the reversed hydrolysis reaction of *Diplococcus* enzyme, the synthesis of alkyl-disaccharide hasn't been reported (12). So far as I examined, *Diplococcus* enzyme couldn't transfer the disaccharide from *p*NP-substrate to 1-alkanols in the presence of sodium cholate (data not shown). In contrast, since the *Bacillus* enzyme has relatively broad specificity for aglycone and greater stability in organic solvents such as 15 % (v/v) methanol, ethanol, acetone and dimethyl sulfoxide (data not shown), the enzyme seems to be suitable for synthesis of neo-glycolipid. In deed, *Bacillus* enzyme could transfer the disaccharide from *p*NP-substrate to various water-soluble alcohols at a transfer ratio of 70 % or more. Although the transfer ratios were lower for water-insoluble alcohols than soluble ones, they efficiently increased when the detergent, sodium cholate, was added to the reaction mixtures. Furthermore, the *Bacillus* enzyme could directly transfer the disaccharide from native glycoprotein, asialo-fetuin, to 1-alkanols. Synthesis using *p*NP-disaccharide as the glycosyl donor is impractical because of the cost, however the method using glycoprotein seems to be valuable. As the enzyme was a retaining type, the disaccharide was selectively transferred to the acceptor in the α-configuration. It is difficult to introduce GalNAc to an alcoholic hydroxyl group in the α-configuration by organic chemical methods because of interference by the *N*-acetyl group of the GalNAc C-2 position. Therefore, enzymatic synthesis using *Bacillus* enzyme is very effective. As the enzymatically synthesized hexyl-GalNAc-Gal efficiently inhibited the binding of anti-T antigen antibody to asialo-fetuin, it has potential as an agent for blocking T antigen-mediated cancer metastasis.

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Section 4. Trypsin Inhibitory Activity of Bovine Fetuin De-*O*-glycosylated by Endo-α-*N*-acetylgalactosaminidase

INTRODUCTION

Mucin-type oligosaccharides, the major class of *O*-glycan, occur in various animal glycoproteins and are characterized by the core structure of Galβ1→3GalNAcα-Ser/Thr (1). Frequently, mucin-type oligosaccharides are found in serine- and threonine-rich peptides of mucoid or membrane-bound proteins at high densities, and these oligosaccharides provide physical stability and resistance against environmental factors such as acids and proteases. The binding affinity of some lectins or antibodies to high-density mucin-type oligosaccharides is known to be much higher than that of low-density oligosaccharides (2). On the other hand, mucin-type oligosaccharides were also found sparsely in soluble glycoproteins. The biological function(s) of these sugar chains are poorly understood (3). Although, serum glycoprotein hormones such as erythropoietin (4) and β-human chorionic gonadotropin (5) have one and four *O*-glycans per a molecule, respectively, their physiological role was not known.

Fetuin is one of the major compounds of fetal bovine serum proteins and has four *O*-glycans per a molecule (6–7). Several biological functions of fetuin have been reported as follows: lymphocyte-stimulating property, lipid-binding capacity, binding of thyroid hormone, stimulating growth and adherence of cultured cells, and opsonization (8–10). Furthermore, fetuin has significant homology with a kind of trypsin inhibitor and its protease inhibitory activity was reported (11–12). Some of these functions of fetuin were considered to involve its strong negative charge because of the presence of *N*-acetylneuraminic acid residues at the non-reducing terminals of *O*- and *N*-glycans. However, the relationship between biological functions and neutralized *O*-glycan of fetuin is unknown.

In the Section 1 of Chapter II, I described the purification of endo-α-*N*-acetylgalactosaminidase (endo-α-GalNAc-ase, EC 3.2.1.97) from *Bacillus* sp., which hydrolyzes *O*-linked disaccharide, Galβ1→3GalNAc, bound to serine or threonine of glycoproteins in the α-configuration (13). The *Bacillus* enzyme had a much higher hydrolysis rate for asialo-fetuin (de-sialylated fetuin) than two commercially available endo-α-GalNAc-ases, from *Diplococcus pneumoniae* (14–15) and *Alcaligenes* sp. (16). In this chapter, I attempt to elucidate the function of *O*-glycan in bovine fetuin using *Bacillus* endo-α-GalNAc-ase.

MATERIALS AND METHODS

Materials. Bovine fetuin (F3004) and POD conjugated goat anti-IgM antibody were purchased from Sigma. Neuraminidase from *Arthrobacter ureafaciens* was from Nacalai Tesque, Japan. Endo-α-GalNAc-ase from *Bacillus* sp. was purified as described in Section 1 of Chapter II (12). Mouse anti-T (Thomsen-Friedenreich) antigen monoclonal antibody was from DAKO. Porcine pancreas trypsin and POD immunostain kit were from Wako Pure Chemicals, Japan. Benzoil-L-arginine *p*-nitroanilide (L-BAPA) was from Peptide Institute Inc., Japan. Block Ace was from Dainippon Pharmaceutical, Japan.

Measurement of protein. The protein concentration was measured using BCA protein assay reagent (Pierce) with bovine serum albumin as the standard.

Trypsin inhibitor assay. The trypsin inhibitory activity was measured in terms of the decrease in trypsin activity after incubation with fetuin or its derivatives. The reaction mixture composed of 1.0 μg/ml trypsin and suitable amount of inhibitor in 50 mM Tris-HCl buffer (pH 7.5) was previously incubated at 25 °C for 2 min. Then, L-BAPA was added to 0.1 mM and released *p*-nitroanilide was monitored by 405 nm at the same temperature (16).

SDS-PAGE and western blotting. SDS-PAGE was performed in 10 % polyacrylamide with a discontinuous Tris-glycine buffer (pH 8.5) containing 0.1 % SDS. Fetuin and its derivatives (7.8 μg each) were subjected to SDS-PAGE and electroblotted to nitrocellulose membrane (Bio-Rad Lab.). The membrane was immunostained using mouse anti-human Thomsen-Friedenreich antigen monoclonal antibody (DAKO-HB-T1, 3.7 μg/ml), POD-conjugated goat anti-mouse IgM (Sigma, 3.5 μg/ml) and POD immunostain set (Wako pure chemical, Japan) containing nitrotetrazolium blue as the chromogen. After blotting, the remaining gel was stained using Coomassie Brilliant Blue R-250.

CD analysis. CD spectra of fetuin and its derivatives were measured with a spectropolarimeter Model J-720 (JASCO, Japan). The temperature of the solution in the cuvette was controlled at 25 °C. The path length of the optical cuvette was 2.00 mm. All protein solutions contained 78 μg/ml in 10 mM Tris-HCl buffer (pH 7.5).

RESULTS AND DISCUSSION

Preparation of asialo- and de-O-glycosylated fetuin

To prepare asialo- and de-*O*-glycosylated fetuin, 5 mg of fetuin was incubated in 10 mM sodium acetate buffer (pH 5.0) at 37 °C for 24 h with 0.2 units of neuraminidase (from *Arthrobacter ureafaciens*), and with 0.2 units of neuraminidase and 0.1 unit of highly purified *Bacillus* endo-α-GalNAc-ase, respectively. After dialysis against 10 mM Tris-HCl buffer (pH 7.5), both reaction mixtures were analyzed by SDS-PAGE. As shown in Fig. 1, neuraminidase treatment decreased the molecular mass of fetuin by about 4000 Da. As fetuin contains about 13 residues of *N*-acetylneuraminic acid at the non-reducing terminals of both *O*- and *N*-glycans, the reduction of molecular mass by neuraminidase treatment corresponds to the theoretical value. Both treatment of neuraminidase and endo-α-GalNAc-ase also reduce the molecular mass of fetuin, but the difference in mobility from asialo-fetuin obtained by neuraminidase treatment was not unclear on SDS-PAGE. Then, to confirm the removal of *O*-linked Galβ1→3GalNAc from asialo-fetuin by endo-α-GalNAc-ase, western blotting analysis was carried out using mouse anti-human Thomsen-Friedenreich antigen (T antigen) monoclonal antibody (DAKO-HB-T1) which recognize the Galβ1→3GalNAcα structure. As a result, neuraminidase-treated fetuin (asialo-fetuin) gave a few positive bands, and native fetuin also gave a few weak bands, but the neuraminidase- and endo-α-GalNAc-ase-treated fetuin scarcely reacted with anti-T antigen antibody. These results indicate that the removal of *O*-linked disaccharide from asialo-fetuin by endo-α-GalNAc-ase seemed to be almost complete.

CD analyses of fetuin preparations

To examine the structure of de-*O*-glycosylated fetuin, CD spectrum analysis was carried out using a spectropolarimeter Model J-720 (Jasco). The protein solutions were analyzed in the concentration of 78 μg/ml in 10 mM Tris-HCl buffer (pH 7.5). As the result, no difference between asialo- and de-*O*-glycosylated fetuin was observed, but native fetuin was slightly different from the other two samples (data not shown). These results suggested that *N*-acetylneuraminic acid residues at the non-reducing end of sugar chains have some effect on the three-dimensional structure of fetuin, but *O*-linked disaccharides don't affect it.

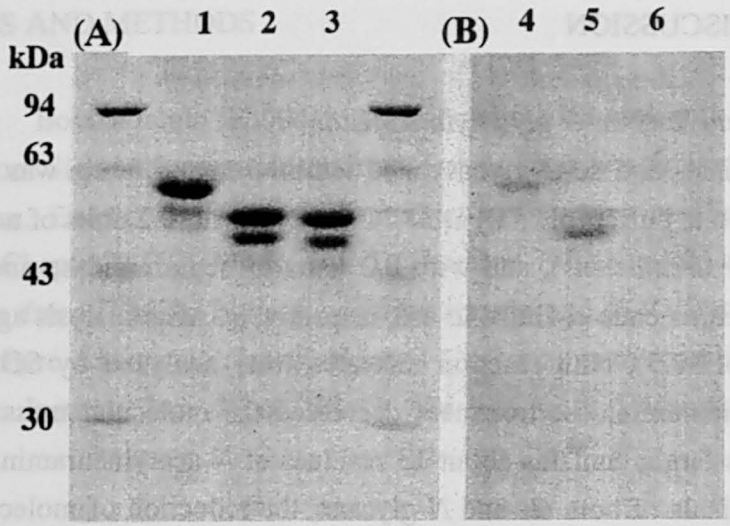

Fig. 1. SDS-PAGE (A) and western blotting (B) of native, de-sialylated and de-*O*-glycosylated forms of fetuin.

SDS-PAGE was performed in 10 % polyacrylamide gel with a discontinuous Tris-glycine buffer (pH 8.5) containing 0.1 % SDS. Native fetuin and its derivatives (7.8 μg each) were subjected to SDS-PAGE and electroblotted to nitrocellulose membrane. The membrane was immunostained using mouse anti-human T antigen monoclonal antibody (3.7 μg/ml), POD-conjugated goat anti-mouse IgM (3.5 μg/ml) and POD immunostain kit containing nitrotetrazolium blue as the chromogen. After blotting, the remaining gel was stained using Coomassie Brilliant Blue R-250. Lanes 1 and 4: native fetuin; lanes 2 and 5: de-sialylated fetuin; lanes 3 and 6: de-*O*-glycosylated fetuin; M: marker proteins.

Trypsin inhibitory activities of fetuin preparations

Trypsin inhibitory activities of these fetuin preparations were examined by incubating them with porcine pancreas trypsin, followed by the assay of the remaining trypsin activity. The reaction mixture composed of 1.0 μg/ml trypsin and a suitable amount of fetuin preparation in 50 mM Tris-HCl buffer (pH 7.5) was previously incubated at 25 °C for 2 min. Then, 0.1 mM L-BAPA was added and the released *p*-nitroanilide was determined by monitoring at 405 nm at the same temperature (16). As shown in Fig. 2, trypsin activity decreased with an increasing amount of native fetuin added, and finally reached complete inhibition at about a 1:1 molar ratio of fetuin and trypsin. In contrast to the result of native fetuin, the trypsin inhibition by asialo-fetuin was about 2-fold less effective. Interestingly, de-*O*-glycosylated fetuin decreased the trypsin inhibitory activity to about one-third of that of native fetuin (although asialo- and de-*O*-glycosylated fetuin preparations contained a small amount of neuraminidase

and endo-α-GalNAc-ase, these glycosidases didn't have any trypsin inhibitory activity). These results suggest that in addition to *N*-acetylneuraminic acid, *O*-linked disaccharide, Galβ1→3GalNAc, plays some significant role in trypsin inhibitory activity of fetuin.

The attachment sites for *O*-glycan in fetuin are Ser-253, Thr-262, Ser-264 and Ser-278 (6–7), and their surrounding region is proline-rich and relatively hydrophobic. Dziegielewska *et al.* (8) suggested that these oligosaccharides might prevent its aggregation and maintain solubility of the fetuin, though our CD analysis showed very few differences between native fetuin and de-*O*-glycosylated fetuin. Ishii-Karakasa *et al.* (18) reported that fetuin contained a small amount of branched *O*-glycan, Galβ1→3(Galβ1→4GlcNAcβ1→6)GalNAc, which could not be hydrolyzed by the *Bacillus* endo-α-GalNAc-ase. Although *O*-glycan could not be completely removed from asialo-fetuin by the *Bacillus* endo-α-GalNAc-ase, the trypsin inhibitory activity decreased. From this result, I suppose that the *O*-linked oligosaccharides or their attachment region may be involved in the trypsin inhibitory activity of fetuin.

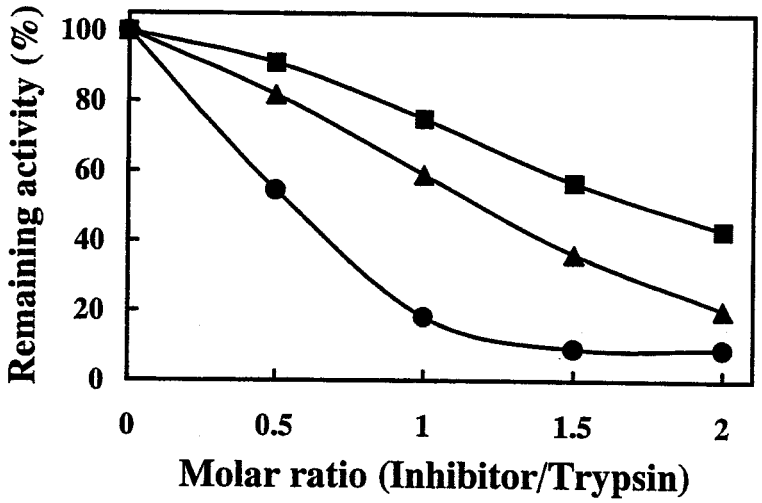

Fig. 2. Effects of native, de-sialylated and de-*O*-glycosylated forms of fetuin on trypsin activity.

The reaction mixture composed of 1.0 μg/ml trypsin and a suitable amount of various fetuin preparations in 50 mM Tris-HCl buffer (pH 7.5) was previously incubated at 25 °C for 2 min. Then, 0.1 mM L-BAPA was added and released *p*-nitroanilide was determined by monitoring at 405 nm. Each point represents the mean of six determinations. Symbols: ●, native fetuin; ▲, de-sialylated fetuin; ■, de-*O*-glycosylated fetuin.

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Summary of Thesis

Chapter I.

α-*N*-Acetylgalactosaminidase (α-GalNAc-ase; EC 3.2.1.49) is an exoglycosidase specific for the hydrolysis of terminal α-linked *N*-acetyl-D-galactosamine (GalNAc) in various sugar chains. The gene, *nagA*, encoding α-GalNAc-ase from *Acremonium* sp. was cloned, sequenced, and expressed in yeast *Saccharomyces cerevisiae*. The *nagA* gene contains an open reading frame which encodes for 547 amino acid residues including 21 residues of a signal peptide in its *N*-terminal. The calculated molecular mass of mature protein from the deduced amino acid sequence of *nagA* is 57260 Da, which corresponds to the value obtained from SDS-PAGE of native enzyme. NagA showed significant homology to eucaryotic α-GalNAc-ases and α-galactosidases (α-Gal-ases), particularly α-Gal-ase A (AglA) from *Aspergillus niger*. Phylogenetic analysis revealed that NagA didn't belong to the cluster of vertebrate α-GalNAc-ase and α-Gal-ase but formed another cluster with AglA and yeast α-Gal-ases. Thus the evolutionary origin of the fungal α-GalNAc-ase was suggested to be different from that of vertebrate α-GalNAc-ase. This is the first report of a microbial α-GalNAc-ase gene.

Chapter II.

Section 1.

Endo-α-*N*-acetylgalactosaminidase (endo-α-GalNAc-ase, EC 3.2.1.97) was purified to homogeneity from the culture fluid of *Bacillus* sp. isolated from soil, and characterized. The molecular mass of the enzyme was estimated as 110 kDa. The enzyme was stable at pH 4.0–10.0, up to 55°C, and was most active at pH 5.0. The substrate specificity of the enzyme was strict for the disaccharide, galactosyl β1, 3 *N*-acetyl-D-galactosamine (Galβ1→3GalNAc), bound to aglycone in α configuration. On the other hand, the specificity of the enzyme for the aglycone structure was fairly relaxed.

Section 2.

Endo-α-GalNAc-ase from *Bacillus* sp. could transfer the disaccharide, Galβ1→3GalNAc, from *p*-nitrophenyl substrate to various acceptors, such as monosaccharides, disaccharides and sugar alcohols. The transfer ratios were 29–36 % for monosaccharides, 14–17 % for disaccharides, and 24–25 % for sugar alcohols. Using this

transglycosylation activity of the endoglycosidase, it was possible to synthesize various neo-oligosaccharides.

Section 3.

Thomsen-Friedenreich antigen (T antigen) disaccharide, Galβ1→3GalNAc, containing glycolipid mimicry were synthesized using the transglycosylation activity of endo-α-GalNAc-ase from *Bacillus* sp. The enzyme could transfer the disaccharide from *p*-nitrophenyl substrate to water-soluble 1-alkanols and other alcohols at a ratio of 70 % or more. Although the transfer ratios were lower for water-insoluble alcohols than soluble ones, they were increased by adding sodium cholate to the reaction mixtures. The enzyme also transferred the disaccharide from asialo-fetuin to 1-alkanols directly. The anomeric bond between the disaccharide and 1-alkanols of the transglycosylation product was an α-configuration as determined by sequential digestion of exo-glycosidases. As the transglycosylation product, hexyl-GalNAc-Gal, efficiently inhibited the binding of anti-T antigen mono-clonal antibody to asialo-fetuin, it has potential as an agent for blocking T antigen-mediated metastasis of cancer cells.

Section 4.

The effect of bovine fetuin *O*-glycans on its trypsin inhibitory activity was examined. De-sialylated (asialo-) and de-*O*-glycosylated fetuin were prepared from native fetuin using *Arthrobacter* neuraminidase and the mixture of it and *Bacillus* endo-α-*N*-GalNAc-ase, respectively. De-sialylation and de-*O*-glycosylation from fetuin were confirmed with SDS-PAGE followed by western blotting using anti-human Thomsen-Friedenreich antigen (T antigen) antibody which recognizes *O*-linked Galβ1→3GalNAc. Native fetuin completely inhibited the trypsin activity at about 1:1 molar ratio. In contrast, the trypsin inhibitory activity of asialo- and de-*O*-glycosylated fetuin decreased about a half and one third of that of native fetuin, respectively.

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芦田久

Hisashi Ashida

List of Publications

Publications related to this thesis

Hisashi Ashida, Hisanori Tamaki, Kenji Yamamoto, and Hidehiko Kumagai, 2000. Molecular Cloning of cDNA Encoding α-*N*-Acetylgalactosaminidase from *Acremonium* sp. and Its Expression in Yeast. *Arch. Biochem. Biophys.* in press.

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Hisashi Ashida, Kenji Yamamoto, Hidehiko Kumagai, and Tatsurokuro Tochikura, 1992. Purification and Characterization of Membrane-bound Endoglycoceramidase from *Corynebacterium* sp. *Eur. J. Biochem.* **205**, 729–735.

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